**Universidade de Aveiro** Departamento de Biologia
**2012**

**Cláudio Alexandre Tabaio Brandão**

# Novas perspetivas do ciclo de vida e interações em *Symbiodinium* spp

# New insights into the *Symbiodinium* life cycle and strain interactions

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – Ramo de Biologia Marinha, realizada sob a orientação científica do Doutor Jörg Christian Frommlet, investigador da Universidade de Aveiro e do Professor João António de Almeida Serôdio, Professor auxiliar do Departamento de Biologia da Universidade de Aveiro

Este trabalho foi financiado por fundos nacionais através da FCT – Fundação para a Ciência e Tecnologia, no âmbito do projecto SeReZoox (referência FCT: PTDC/MAR/113962/2009)

Mar Português

“Ó mar salgado, quanto do teu sal
São lágrimas de Portugal!
Por te cruzarmos, quantas mães choraram,
Quantos filhos em vão rezaram!

Quantas noivas ficaram por casar
Para que fosses nosso, ó mar!
Valeu a pena? Tudo vale a pena
Se a alma não é pequena.

Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu.”

Fernando Pessoa, em “Mensagem”.

Aos meus pais, irmã, avós e bons amigos, que, ao longo da minha vida me tornaram uma pessoa melhor.

**o júri**

presidente

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**agradecimentos**

First of all, I would like to thank in general to everyone that made this thesis possible.

It is a pleasure to thank my main supervisor Jörg Frommlet and my co-supervisor Joâo Serôdio without whom this work would not have been possible. In particular, I would like to thank Jörg for his infinite patience and for everything that he has done for me.

To the Lab 8.2.6, “Phycology Lab”, where I spent a lot of time with great people that contributed in several ways to my knowledge and scientific career. Thank you guys!

To all of my friends. In particular, to Diogo Moreira and Tiago Pereira, the best friends one could have! Also to Jennie Gil and Rita Silva, for all the good moments we spent together! To Nádia and Luana, the greatest acquisitions to my friends list over my graduation! Thank you all! =)

To my family. In particular to my parents, Fausto José Neves Brandão and Maria de Lurdes Carrilho Tabaio Brandão, who have allways done the impossible for me. To my big sister, Susana Brandão, for allways been there. To my grandmother “Avó Júlia” for everything she means to me. And last, but not least, to my grandmother “Avó Nélita”, for being the role model I allways try to follow, I miss you ans I allways will!

To Carina, because… she knows why =D

To everyone that is not described in here!

**palavras-chave**

Corais, Symbiodinium, ciclo de vida, gametogénese, espécies reactivas de oxigénio, competição.

**resumo**

*Symbiodinium* spp. é uma espécie de dinoflagelado que tem a capacidade de estabelecer uma relação mutualística com diversos invertebrados marinhos. A simbiose que *Symbiodinium* spp. estabelece com os corais é de extrema importância ecológica e socioeconómica, porém, nas últimas décadas, os corais têm vindo a sofrer cada vez mais agressões antropogénicas. Com o objectivo de se prever a resiliência dos corais num ambiente em mudança, é vital que se conheça na íntegra ambos os parceiros simbióticos, assim como as relações que estes estabelecem entre si. Duas das lacunas no estado da arte acerca da biologia e ecologia de *Symbiodinium* spp. são: i) a falta de informação sobre a sua história de vida, nomeadamente sobre a existência de reprodução sexuada e ii) a falta de informação sobre as interacções entre as células de *Symbiodinium* spp. Este estudo teve como objectivo abordar estes dois aspectos, utilizando, de modo a cumprir os objectivos, culturas monoclonais de *Symbiodinium* spp. Através do recurso a diversas técnicas laboratoriais, tais como: microscopia convencional e de epifluorescência, citometria de fluxo e experiências de cruzamento com diferentes estirpes de *Symbiodinium* spp., várias fases do ciclo de vida ainda não descritas foram identificadas e documentadas pela primeira vez. Relativamente às interacções entre as estirpes de *Symbiodinium* spp., verificou-se que, ao contrário do que se esperava, a espécie que se comporta como agressora sofreu um maior impacto ao nível do seu crescimento. Contudo, ambas as culturas utilizadas nas experiências de cruzamento revelaram ser afectadas pela presença do outro competidor.

**keywords** corals, *Symbiodinium*, life cycle, gametogenesis, reactive oxygen species, competition.

**abstract**

*Symbiodinium* is a dinoflagellate that establishes a mutualistic relationship with a range of marine invertebrates. The symbiosis between *Symbiodinium* spp. and corals is of particular ecological and socio-economical importance. Over the last decades, corals have been suffering increasingly from anthropogenic stressors. In order to predict the resilience of corals in a changing environment, detailed knowledge on the symbiotic partners and their complex interactions is vital. Two critical gaps in the knowledge on *Symbiodinium* biology and ecology are: i) incomplete information on its life history, especially on its sexual reproduction and ii) lack of information on direct cell-cell interactions, which are commonly found in free-living dinoflagellate species. This study aimed at addressing these two aspects in cultures of *Symbiodinium* spp. grown ex situ. For this, a collection of clonal cultures was established. Based on light/ epifluorescence microscopy and crossing experiments between different strains from this collection, several new life cycle stages could be identified and first direct observational evidence for a sexual life cycle in *Symbiodinium* spp. could be found. Experiments on cell-cell interactions revealed different interactions at the inter- and intra-cladal level and between different life cycle stages. In one pair of interacting strains, the effects of direct cell-cell contact on growth characteristics were studied by flow cytometry. Generally, strain interactions had positive effects on growth rates but negative effects on carrying capacity. However, the strains in the co-culture were not affected equally by the presence of the competing strain.

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# 1. Introduction

## 1.1. Corals: A symbiosis between cnidarian host and algal symbiont

Coral reefs are unique and extremely diverse ecosystems, matched on land only by tropical rain forests. At the basis of these ecosystems are corals, which have shaped these ecosystems in tropical oceans over the last 200 million years (Hoegh-Guldberg, 1999). Corals are animals that belong to the Phyllum Cnidaria, Class Anthozoa. They are among the simplest animals on earth, having only two tissue layers (Douglas, 2003). There are two main types of corals, hard corals (or scleractinian corals) and soft corals. The scleractinian corals are able to secret calcium carbonate on the outside of their polyps, building hard structures in which many polyps live together and that build the basis for coral reefs. Soft corals do not secrete calcium carbonate and thus they do not contribute to the reef structure in the same extent as scleractinian corals. Their polyps are connected by fleshy tissue and additional structure is given to their colonies by calcite spicules (scerites) that are produced within their tissue (Jeng et al., 2011; Konishi, 1981).

Both hard and soft corals are able to host symbiotic, unicellular algae inside their endodermal/gastrodermal cells (Figure 1). Within these cells, the algae are surrounded by specific structures called symbiosomes (Wakefield et al., 2001). The symbiotic algae belong to the dinoflagellate genus *Symbiodinium* and, when in hospite, are also referred to as zooxanthellae - a non-taxonomic term that describes coccoid yellow-brown endosymbionts that can be dinoflagellates or diatoms (Trench, 1979, 1997; Blank and Trench, 1986, Trench and Blank, 1987, Banaszak et al., 1993). There are numerous other organisms that host *Symbiodinium* in a mutualistic relationship, such as molluscs, nudibranchs, platyhelminths, foraminiferans and sponges (Freudenthal, 1962; Hill et al., 2011; Pawlowski et al., 2001; Rogers, 2011). However, the symbiosis between corals and *Symbiodinium* is of particular ecological and economic importance because of its crucial importance for coral reef ecosystems (Hoegh-Guldberg et al., 2007).

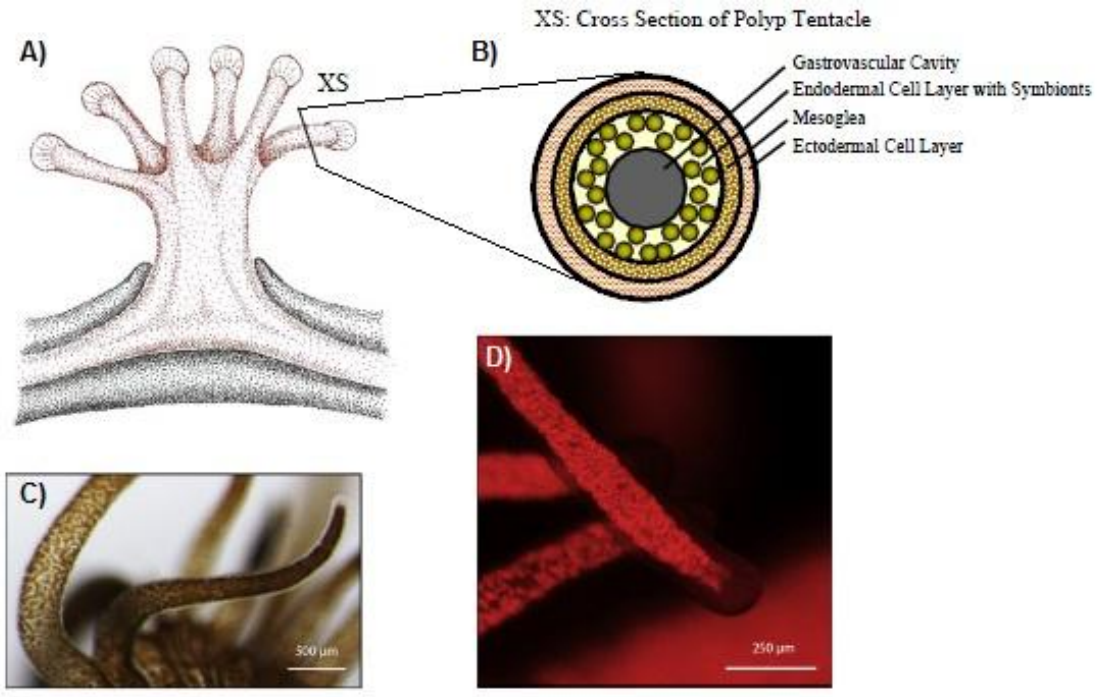


**Figure 1 -** On top, an illustration of a typical zoanthid (A) and a cross section of a polyp tentacle and the location of the endosymbionts (B). Adapted from Hauck (2007). Bellow, photographs of the tentacles of *Aiptasia pallida*. C) detail of the patchy distribution of the endosymbionts; D) clorophyll autofluorescence of the endosymbionts, corroborating their localization inside gastrodermal cells. Adapted from Fransolet et al. (2012).

When *in symbio*, the symbiont provides the host with up to 95% of its energy requirements (Kleypas et al., 1999; Muscatine, 1990). The photosynthetically fixed carbon that is produced by the algae is translocated to the host in form of glucose, glycerol and amino acids. The control over the translocation process is still not fully understood but host release factors seem to play an important role (Yellowlees et al., 2008). The symbiosis also provides the host with elevated concentrations of oxygen, which accelerates higher mitochondrial respiration rates, thus resulting in high ATP levels that supports the rapid deposition of calcium carbonate in the calcification process (Colombo-Pallotta et al., 2010; Jokiel, 2011). In exchange, *Symbiodinium* receives a safe environment as well as waste inorganic nutrients from the host, such as ammonia, phosphate and carbon dioxide, which are all essential substrates for photosynthesis (Yellowlees et al., 2008). Thus, the close symbiosis between cnidarians and *Symbiodinium* allows for very efficient nutrient cycling; a major feature that

ensures the success of this mutualistic relationship in the often extremely oligotrophic environments of tropical marine waters.

The symbiosis may vary as an adjustment to changes in the environment to optimize the survival of the holobiont (Fransolet et al., 2012). The prevalent method of acquiring the symbionts is via horizontal transmission, i.e. uptake of free-living *Symbiodinium* from the environmental pool. The coral larvae acquire their symbionts during the feeding process (Harii et al., 2009; Rodriguez-Lanetty et al., 2004; Rodriguez-Lanetty et al., 2006). However, *Symbiodinium* also plays an active role in the acquisition process, as it shows chemotaxic behavior towards the cnidarian host (Pasternak et al., 2004) and phototaxis to remain in a suitable photic zone (Hollingsworth et al., 2005).

Through mutual recognition mechanisms, both host and symbiont are able to recognize each other in order to establish the symbiosis (Figure 2). The host and the symbiont recognize each other and when compatible partners meet, binding occurs. Several studies showed that pattern recognition receptors (e.g. carbohydrate-binding proteins) are involved in the recognition and binding to specific conserved components of the cell walls (e.g. lectins) (Goldstein et al., 1980; Kilpatrick, 2002). In addition, the symbiont is able to disrupt the host cell machinery in order to avoid digestion. Successively, the host-derived vacuole (symbiosome) is formed and the symbiosis is established (Fransolet et al., 2012).

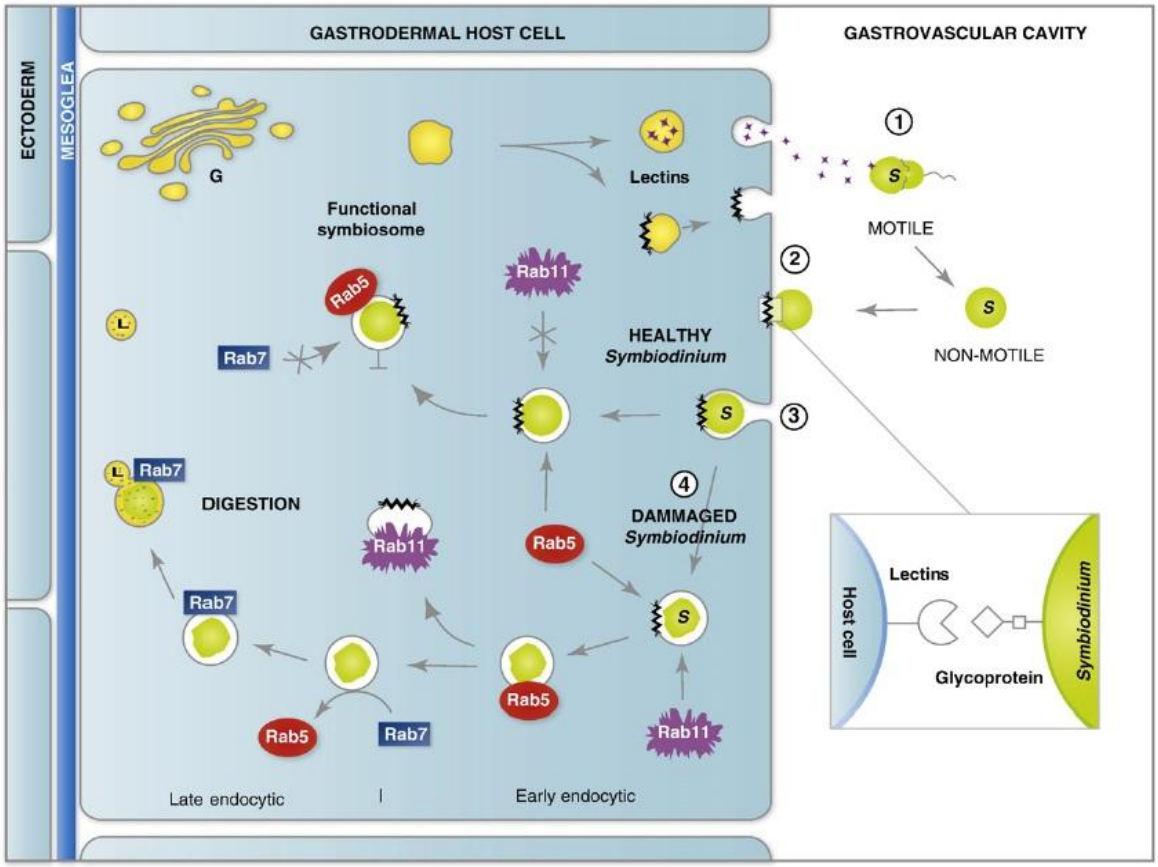


**Figure 2** - Schematic representation of the mechanisms underlying the establishment of the symbiosis between cnidarians and *Symbiodinium* spp. Through the use of specific lectins (1), the host induces the motile algae to revert to the coccoid stage. In (2) the mutual recognition mechanism through specific lectins in the host and glycoproteins in the surface of the symbiont is shown. Phagocytosis of the zooxanthellae takes place (3). Healthy *Symbiodinium* spp. cells end up in the functional symbiosome while damaged cells are digested (4). Taken from Fransolet et al. (2012)

## 1.2. *Symbiodinium*: a symbiotic dinoflagellate

The genus *Symbiodinium* belongs to the family Symbiodiniaceae, order Suessiales, class Dinophyceae, phyllum (division) Pyrrophyta or Dinophyta (Freudenthal, 1962; Taylor, 1974; Fensome et al., 1993; Steidinger and Tangen, 1997). Members of this phyllum are unicellular alveolate protists, closely related to both the apicomplexans and the ciliates. There are approximately 2000 living species in this phylum, forming a monophyletic group (Coats, 2002; Lenaers et al., 1989; Rogers, 2011; Zhang et al., 2000). Based on the fossil record, dinoflagellates developed in the Silurian (Thomas, 1996). Most

extant species are marine (Lee, 2008), but they are also found in freshwater habitats as well as in extreme habitats such as artic and hypersaline environments (Hasle et al., 1996). Dinoflagellates can obtain their energy by means of photosynthesis, heterotrophy or both (mixotrophy) and, as a result, were described taxonomically by both zoologists and botanists (Rogers, 2011). Approximately half of the species are considered obligate phototrophs, but more and more species that initially had been described as obligate phototrophs are found to be mixotrophs. One recent example for the discovery of mixotrophy is *Symbiodinium*, which changes our view of the survival strategies for these symbiotic dinoflagellates (Jeong et al., 2012).

Some dinoflagellate species also establish parasitic, commensal or mutualistic relationship with many invertebrates, including Mollusca, Cnidaria, Platyhelminths, Foraminifera and Porifera (Hill et al., 2011; Pawlowski et al., 2001; Rogers, 2011). Overall, there are eight genera in about four orders that have symbiotic dinoflagellate representatives. *Symbiodinium* spp. is the most common symbiotic dinoflagellate found in symbiosis with marine invertebrates and protists (Trench, 1979; Pawlowski et al., 2001; Yokouchi, 2003). Freudenthal (1962) was the first to isolate *Symbiodinium* from the jellyfish *Cassiopeia xamachana*.

## Morphology

The dinoflagellate cell is typically divided into an epicone and a hypocone (Figure 3) separated by the transverse girdle (or cingulum). When the coccoid form transforms into a mastigote cell, thecal plates are formed as well as both flagella (Figure 3). Perpendicular to the girdle, there is a longitudinal sulcus. Both of the longitudinal and transverse flagellum emerges through the thecal plates, in the area where the girdle and the sulcus meet. The transverse flagellum is usually wave-like and is closely pressed against the girdle while the longitudinal flagellum projects out from the cell (Lee, 2008).

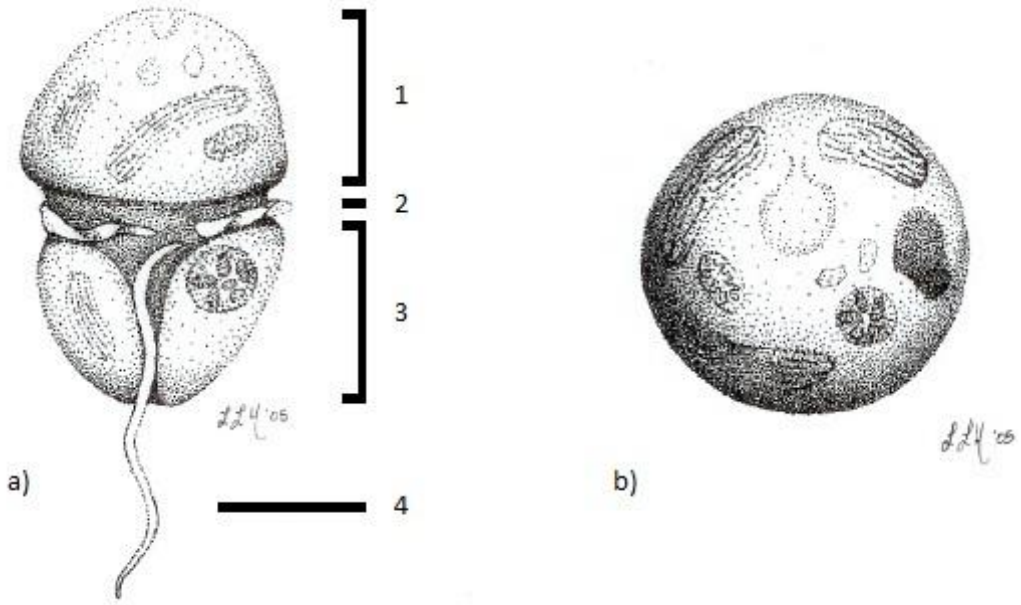


**Figure 3 -** Illustration of dominant forms of *Symbiodinium*. a) motile cell (or mastigote); b) vegetative cell (or cyst); 1 – epicone; 2 – transversal flagellum; 3 – hipocone; 4 – longitudinal flagellum. Adapted from Hauck (2007)

## Taxonomy

When *Symbiodinium spp.* is in symbiosis with its host, the most dominant phase is the phenotypic vegetative form (Stat et al., 2006). At this stage, the cells lack morphological features capable of being identified under the light microscope, so that it was initially thought there was only one pandemic species, *Symbiodinium microadriaticum*. (Freudenthal, 1962; Taylor, 1991). As the ultrastructure and composition of this "species" was studied, it became evident, however, that it is comprised of a highly diverse group of organisms (Schoenberg et al., 1980a, 1980b, 1980c). Different strains have a number of distinct features, namely their chromosome number, vegetative cell size, chloroplast number, size and arrangement, mycosporine-like amino acid production, isoenzyme profile, fatty acids and sterol composition, photoadaptative physiology and host specific infectivity (Banaszak et al., 2000; Blank et al., 1985; LaJeunesse, 2001). However, most of these phenotypic features also show a wide range of plasticity and thus their use as taxonomic

features is limited. For example, factors such as nutrient exposure, irradiance and culture phase affect cell and chloroplast size as well as the biochemical composition of the cells (LaJeunesse, 2001; Rowan et al., 1991; Schoenberg et al., 1980a, 1980b, 1980c). There are also some limitations regarding the culturing of these symbiotic algae isolated from their host, leading to different dominating strains in culture compared to the ones that dominate *in symbio* (Goulet et al., 1997; Santos et al., 2001; Trench, 1979)).

Within the genus, *Symbiodinium spp.* are classified into a number of large groups or clades from A to H (Coffroth et al., 2005) and a recently discovered clade I (Hill et al., 2011; Pochon et al., 2010). Despite the difficulties in their systematics, it is apparent that *Symbiodinium* spp. is much more diverse than it has been described until now and some species have already been identified, including *S. microadriaticum, S. pilosum, S. kawagutii, S. goreaui, S. californium, S. corculorum, S. meandrinae, S. pulchrorum, S. bermudense, S. cariborum, S. muscatinei* (Stat et al., 2006).

## Clades

A vast level of diversity within *Symbiodinium* spp. was found after molecular studies were performed (Baker, 2003; LaJeunesse, 2001; Santos et al., 2001). These studies consisted first in DNA/DNA hybridization and allozymes analyses (Blank and Huss, 1989; Schoenberg et al., 1980a). The hybridization studies showed that the degree of binding among DNAs from several *Symbiodinium* isolates differed as much as DNA from algae in different classes. Consequently, the existent diversity among *Symbiodinium spp.* was placed in an evolutionary context using sequences derived from the nuclear small subunit ribosomal DNA (18S-rDNA) (Rowan et al., 1991). Once again, an incredibly high variability was found, comparable to that of orders of free-living dinoflagellates (Rowan et al., 1992). Consequently, this discovery led to the adoption of a classification scheme for *Symbiodinium spp.* to divide the genus into one of several large groups (or clades). The existing clades are A, B, C (Rowan et al., 1991), D (Carlos et al., 1999), E (LaJeunesse, 2001; LaJeunesse

et al., 2000), F (LaJeunesse, 2001), G (Pawlowski et al., 2001), H (Pochon et al., 2004) and I (Pochon et al., 2010).

Molecular markers

Using DNA sequence analysis, more comprehensive and effective phylogenetic studies could be done, comparing to conventional taxonomic analysis on morphology characteristics. In 1991 the DNA sequence diversity of the small subunit (SSU rDNA) of *Symbiodinium* was described (Rowan et al., 1991). Thus, it was found that the divergent lineages characteristic of the genus, named in the literature as clades, types, phylotypes or groups (LaJeunesse, 2001). Yet, this conserved DNA region is not useful to distinguish at the species level (Baker et al., 1997; McNally et al., 1994; Rowan et al., 1991).

In order to fulfill this gap, attention was changed to the large subunit DNA region (Baker et al., 1997; Lenaers et al., 1991; Thomas P. Wilcox, 1998a) though it was also noticed that the large subunit rDNA wasn't good enough to sort out the differences between genetic types of the major clades (Baker, 1999). Internal transcribed space (ITS) analysis was then used to make the distinction at the species level, once that it has a high degree of variation. In fact, specific regions of the DNA have been explored in order to establish the phylogenetic characterization of *Symbiodinium*, such as small subunit 18S rDNA (McNally et al., 1994; Carlos et al., 1999; Goulet, 1999; Darius et al., 2000), large subunit n28S rDNA (Baker, 1999; Pawlowski et al., 2001) and internal transcribed spacer ITS rDNA (LaJeunesse, 2001).

## 1.3. *Symbiodinium* life cycle

### Asexual life cycle

Kawaguti (1944) described for the first time the swimming behavior of coral's symbionts when they are kept out of the host's tissue. It was the first evidence that the organelle-like zooxanthellae were in fact a living symbiotic cell. However, the first proper description of *Symbiodinium*'s life cycle was only made eighteen years later by Freudenthal (1962). He described several morphological stages based on bright-field microscopy and presented an early outline of the life cycle (Figure 4). The vegetative cell (A) is the dominant stage both in culture and in symbio. It is characterized by a haploid, thin-layered cell, ochraceous in coloration. Freudenthal (1962) described also that vegetative cells could form a cyst with a thicker cell wall (B) under conditions he did not described. Though, the vegetative cyst is now usually referred to only as cyst (Dubinsky et al., 2011; Stat et al., 2006). The cyst may turn into a zoosporangium (C), developing into one (D) to four (E) motile cells, or turn into an aplanospore (J), but only when cultures are grown on solid medium or reach late stationary phase (Freudenthal, 1962). Also, cysts may develop two autospores, the new non-motile vegetative daughter cells (F) (Fitt W., 1983; Freudenthal, 1962). When in symbio, the vegetative cell divides by binary fission (I).

*Symbiodinium* alternates diurnally between a motile and a non-motile stage when kept in liquid culture medium. Light-dark conditions (Figure 5) entrain this transition into its diurnal pattern but *Symbiodinium* spp. also has a endogenous biological clock that maintains those circadian cycles (motile/non-motile) for up to seven days in constant darkness (Fitt, 1983).

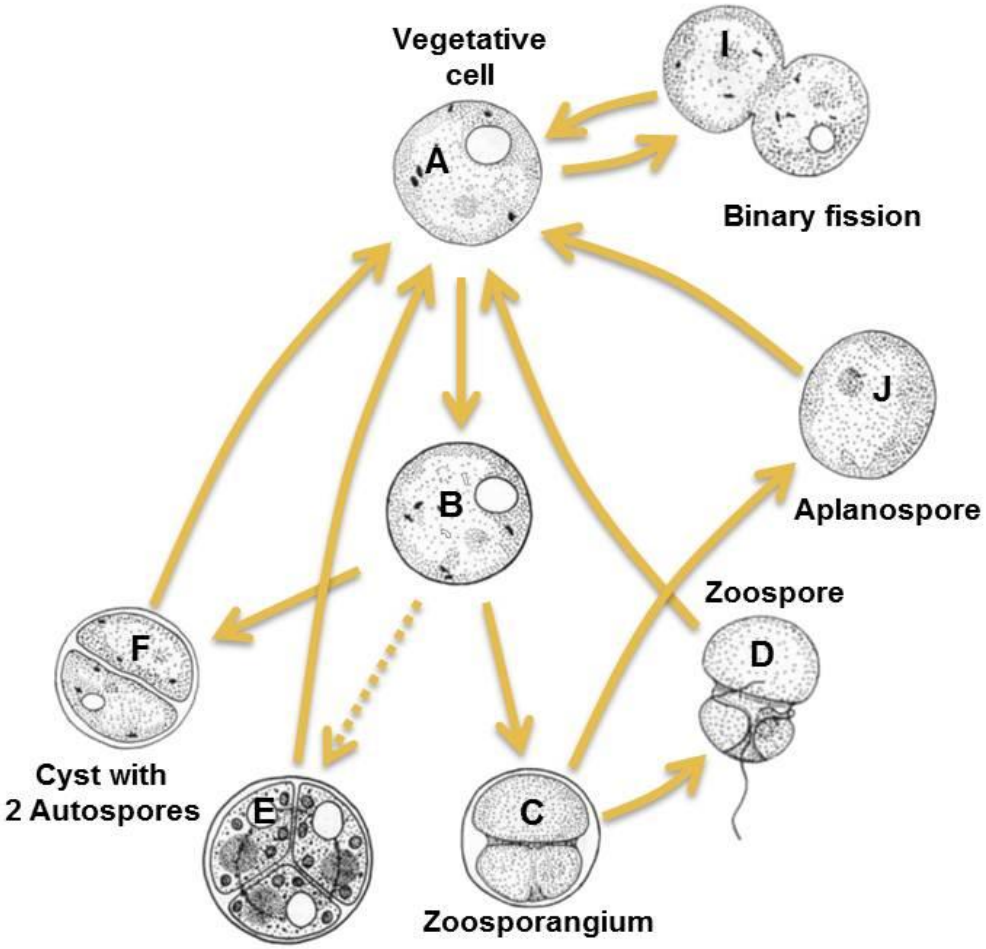


**Figure 4 -** Illustration of *Symbiodinium*'s life cycle as described by Freudenthal (1962). Only asexual stages are represented. A – Vegetative cell, B – Vegetative cyst, C – Zoosporangium, D – Motile zoospore, E – Cyst with three to four developing zoospores, F – Cyst with two new vegetative cells (autospores).

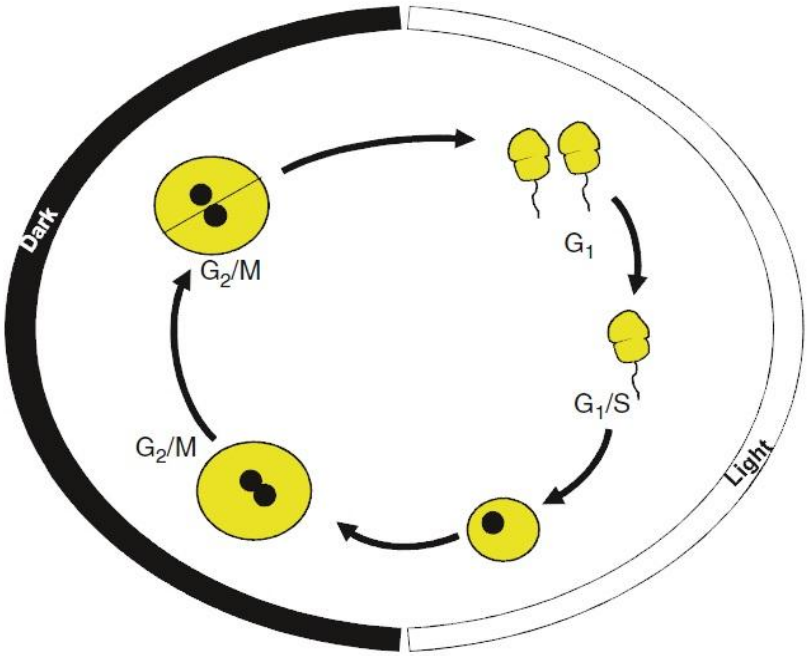


**Figure 5 -** Illustration of the circadian cell cycle of *Symbiodinium spp.* when kept in liquid culture medium with a light:dark cycle. Source: Dubinsky et al. (2011)

*In hospite*

The flagella of *Symbiodinium* spp. cells are not only produced when in free-living conditions, but also when the cells are *in hospite*. However, *in hospite* the flagella are shed or lost strait after mitotic cell divisions (Fitt, 1983). Symbiont densities are controlled by the host, either by controlling their division, or by cell digestion and expulsion (Baghdasarian et al., 2000).

Sexual life cycle

Occasionally, Freudenthal (1962) observed spherical bodies forming in great numbers, which he described as possible gametes (I). These cells showed a faint hint of a girdle. Also, they had a very delicate flagellum and they were described as weak swimmers (Freudenthal, 1962). It was the first indication for sexual reproduction in *Symbiodinium* spp., however, a proof for sexual reproduction (i.e. zoospore pairing) could not be observed.

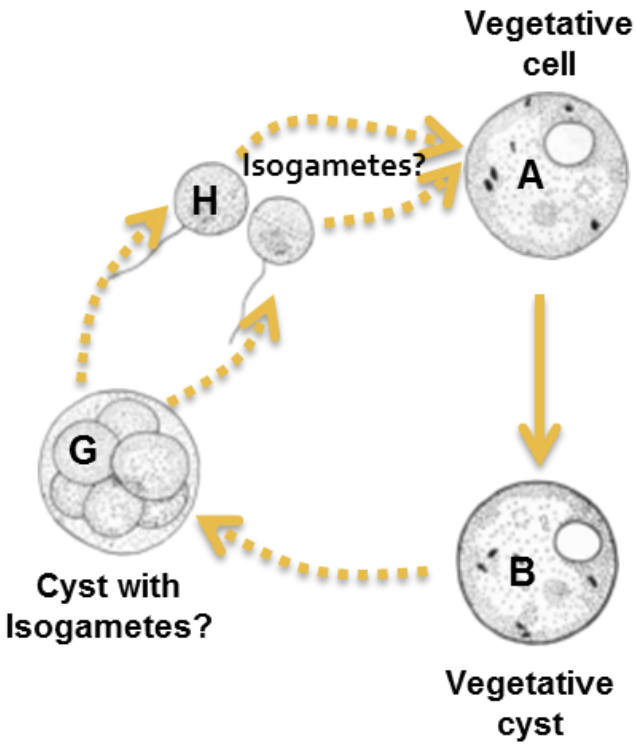


**Figure 6 -** Illustration of *Symbiodinium*'s life cycle. Theoretical sexual stages represented as well as their probable origin. In G, a cell described by Freudenthal (1962) as a possible cyst with developing isogametes, H – putative isogametes.

More recent research provided strong molecular evidence to support the hypothesis of sexual reproduction in *Symbiodinium*. High genetic diversity for isoenzymes, ramdom-amplified polymorphic, and DNA fingerprints, strongly suggests that recombination occurs within *Symbiodinium* populations (Baillie et al., 2000; Goulet et al., 1997; Schoenberg et al., 1980a). If there is recombination happening amongst *Symbiodinium spp.*, high variation levels within and among populations is expected, resulting in more resistant and resilient populations to environmental changes (LaJeunesse, 2001).

## 1.4. Coral Reefs under threat

Coral reef ecosystems provide a number of crucial goods and services of high socioeconomic significance such as tourism, fisheries, coastal protection and drug discovery. Yet, anthropogenic practices such as cyanide and dynamite fishing are jeopardizing these valuable resources, putting their ecological and economic services at risk (Hoegh-Guldberg, 1999). Over the last few decades, anthropogenic stressors such as toxic chemicals (Sánchez-Bayo, 2011), dredging (Erftemeijer et al., 2012) and destructive fishing practices (Cesar et al., 1997) have increased. On top of anthropogenic stressors, natural disturbance events (that are influenced by mankind) such as extended periods of high temperatures and/or disease outbreaks, as well as tropical storms also constitutes a threat to coral reefs (Bert, 2007; Dubinsky et al., 2011; Erftemeijer et al., 2012; Hoegh-Guldberg, 1999; Richmond, 1993). Based on these developments, some of the earlier predictions estimated that coral reefs might disappear entirely within 20 to 50 years (Hoegh-Guldberg, 1999). However, many open questions about the impact of anthropogenic stresses remain and in order to predict the future of coral reefs, it is important to develop a holistic view of the factors and interactions that shape and affect coral reef ecosystems in their entireness.

## 1.5. Coral Bleaching

### The phenomenon

The mutualistic relationship between corals and *Symbiodinium* spp. can be disrupted in several ways. One frequently observed phenomenon is called coral bleaching. It occurs when corals lose their symbiotic algae (through several cellular mechanisms, see figure 2), when the algae lose their pigments or both (Baker et al., 2008; Fitt et al., 1995; Vidal-Dupiol et al., 2009). The term bleaching is used as it refers specifically to the discoloration of corals. The expulsion and/or digestion of the endosymbionts results in the whitening of the

corals, because a big part of a coral's coloration is due to the algal pigments. In the absence of these pigments the carbonate skeleton becomes visible through the transparent coral tissue (Rosenberg et al., 2004).

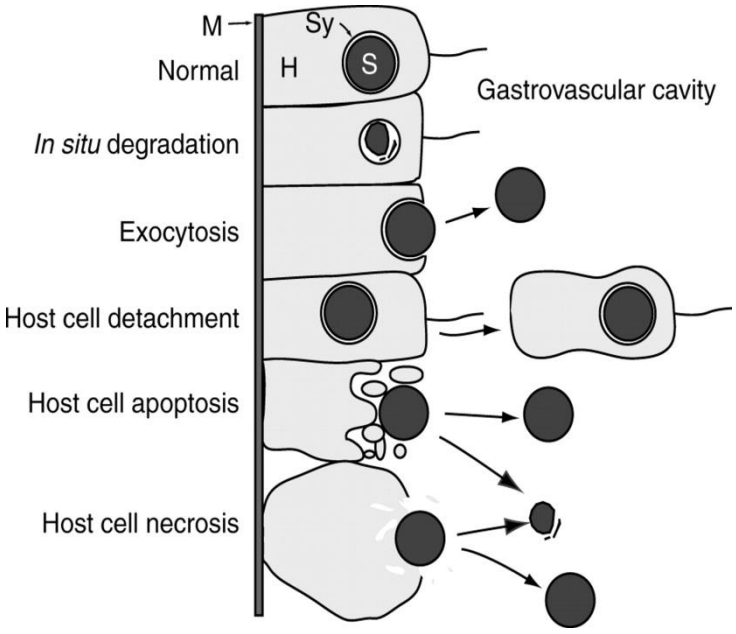


**Figure 7 -** Different cellular mechanisms of bleaching. The symbiotic cells may undergo *in situ* degradation, they may be expelled by the host and the host is able to detach ectodermal cells, together with the zooxanthellae. Host cells undergoing apoptosis or necrosis also contribute to the loss of zooxanthellae. Source: V. M. Weis et al. (2008).

## Causes of bleaching

Atmospheric carbon dioxide ($CO_2$) concentrations have been rising over the last two centuries, leading to both an increase in global average temperatures and an increase in the $CO_2$ uptake by the oceans (Feely et al., 2004). As a consequence of increasing concentrations of carbon dioxide (together with other greenhouse gases such as methane and nitrous oxide), global sea surface temperature is rising at an unprecedented rate (Jansen et al., 2007). In addition, $CO_2$ dissolution in sea water affects the acid/base equilibrium, resulting in the formation of carbonic acid and thus leading to lower carbonate ion concentrations. This, in turn, translates into lower calcification rates by marine calcifying organisms (Jansen et al., 2007; Kleypas et al., 1999; Kleypas

et al., 2008; Wootton et al., 2008). Corals are extremely sensitive to the physicochemical parameters of the surrounding water and even minor variations may cause a breakdown of the obligate symbiosis, having negative consequences for coral survival, growth and reproduction (Hoegh-Guldberg, 1999; Lesser, 2006; Van Oppen et al., 2011). Increased temperature and high levels of irradiance are the main causes for bleaching events (Baird et al., 2009; Feely et al., 2004; Fitt et al., 2001; Hoegh-Guldberg, 1999; Hoegh-Guldberg, 2008; Kleypas et al., 1999; Kleypas et al., 2008; Stat et al., 2006; Vidal-Dupiol et al., 2009). Increased ultra violet light exposure (Fitt and Warner 1995), reduced salinity (Goreau, 1964), and oxidative stress (Lesser, 1997, 2006) can also trigger coral bleaching.

When these parameters deviate too much from the tolerable range, the photosynthetic apparatus of the symbionts can be damaged, resulting in decreased photosynthetic capacity. As a result, the amount of irradiance received by the cells exceeds their capacity of light utilization, causing photoinhibition and the excessive production of reactive oxygen species (ROS)(Figure 8).

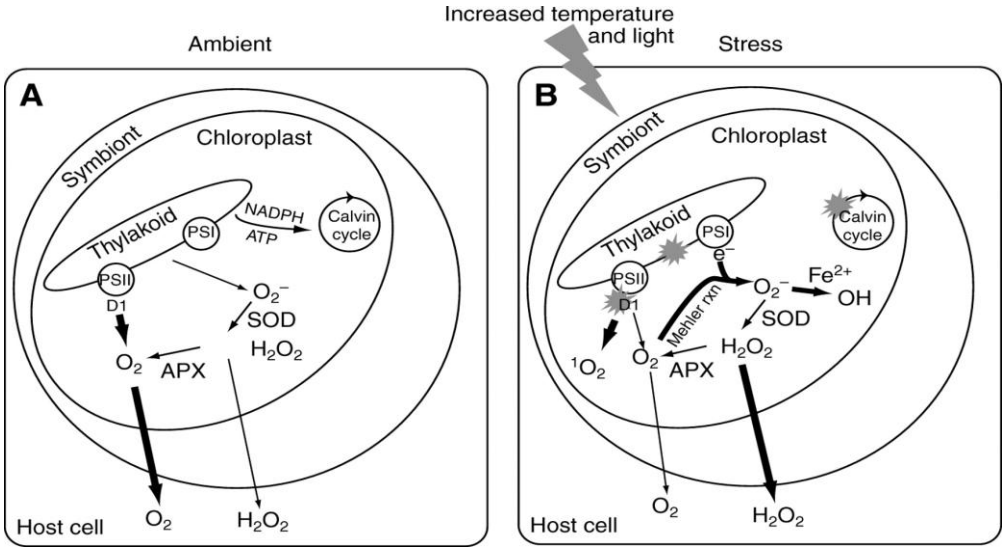


**Figure 8 -** Illustration of the oxygen pathways in *Symbiodinium*. In A, the normal functioning of the pathways is depicted. In B, the cell is under thermal and irradiance stress, leading to malfunctions in the PSII, thylakoid and in the Calvin cycle (marked with a “flash”), resulting in the production of singlet oxygen (1O2) as well as superoxide (O2-). The latter will be converted to the most reactive hydroxyl radical (.OH) and into hydrogen peroxide. Source: Virginia M. Weis (2008).

High production rates of ROS may lead to injury of the photosynthetic apparatus, e.g. the D1 protein complex of photosystem II (PSII), the Calvin cycle and in the thylakoid of the symbiont (figure 3) (Venn et al., 2008). The hydrogen peroxide produced, which is a more stable and diffusible ROS molecule, may also move into host tissues, damaging them and ultimately leading to the expulsion of the symbionts (figure 2) (Fridovich, 1998; Hoegh-Guldberg, 1999; Weis et al., 2008).

For a long time, ROS were regarded only as a toxic by-product of aerobic metabolism, but, more recently, it has become evident that ROS are also involved in a number of metabolic processes such as growth, reproduction, development and stress responses (Aguirre et al., 2005; Finkel, 2003; Halliwell, 2006; Thannickal et al., 2000). Consequently the term “oxidative signaling” has been suggested (Foyer et al., 2005). These signaling and response mechanisms are yet to be understood. How is the signaling through reactive oxygen species identified and which paths are responsible by ROS production are some of the key questions to be answered (Bailey-Serres et al., 2006).

What happens to the coral/symbiont following bleaching?

Following a bleaching event, a coral may survive for weeks to months and the symbiosis may actually recover by re-colonization of the coral with free-living *Symbiodinium*. Research on bleaching has primarily focused on climatological and physiological causes of bleaching as well as on the possible recovery of the symbionts by the host (Brown, 1997; Hoegh-Guldberg, 1999). Yet, the fate of bleached corals was just starting to be broadly studied (Diaz-Pulido et al., 2002). If bleached corals fail to recover their symbionts and stressful conditions prevail long enough, the coral eventually dies and is overgrown by algae, making nearby corals more susceptible to disease and, ultimately, bleaching (Diaz-Pulido et al., 2002).

The resilience of corals to environmental stressors differs between coral species and also depends on the variety of hosted symbiotic clades and their relative densities. The corals that host most ecologically fit *Symbiodinium*

clades show more resilience facing such stressors. The adaptive bleaching hypothesis dictates that when environmental parameters change, symbiotic communities may rapidly vary within a coral, remaining the symbionts that are better adapted to those environmental parameters (Baillie et al., 2000). Consequently, corals improve their resistance and resilience to environmental changes upon the opportunity that a bleaching event provides for them to be repopulated with a better adapted symbiont community, or by shuffling their relative proportions (Baker, 2003; Putnam et al., 2012). This ability of corals to shuffle their symbiotic community and/or to uptake free living *Symbiodinium* may be key to recovery and future survival under changing environmental conditions.

There is a lot of controversy about the viability of the symbionts that are expelled. Some studies claim that the expelled cells are in good physiological shape, therefore contributing to the maintenance of free-living *Symbiodinium* population (Gou et al., 2003; Littman et al., 2008; Pochon et al., 2010; Sandeman, 2006) while others deem that survival of expelled symbionts in the environment is low (Hill et al., 2007; Perez et al., 2001; Steen et al., 1987; Strychar et al., 2004).

Cell-cell interaction

Direct cell-cell interactions between different dinoflagellate species are not uncommon [reviewed in Uchida (2001)]. Some species cause the formation of hypnocysts or temporary cysts in other species (Uchida, 1991, 2001; Uchida et al., 1996) and in extreme cases some dinoflagellates are actually able to kill other species via cell contact (Uchida et al., 1999; Uchida et al., 1995). Additionally, allelopathic interactions (e.g. the production of secondary metabolites that influence the growth, survival and/or reproduction) are also common, showing that different dinoflagellates interact in a whole variety of ways. Often, the outcome of these interactions in the natural environment is not known but it is likely that these interactions strongly influence the population dynamics of the involved species. Cell-cell

interactions between the different clades, types or strains of *Symbiodinium* spp. during the free-living stage of their life cycle could strongly influence their population dynamics, species competition and the outcome of host colonization by these symbiotic dinoflagellates. Yet, this potentially very important aspect of *Symbiodinium* spp. ecology is an entirely unstudied aspect. One focus of this project was to start filling this gap in the knowledge by investigating the cell-cell interactions between different clades and ITS2-types of *Symbiodinium*.

## 1.6. Aims and objectives

A critical gap in the knowledge on *Symbiodinium* biology is the lack of information on the complete life cycle and especially on sexual reproduction (Elbrächter, 2003). Certain aspects of the sexual life cycle in *Symbiodinium* spp. had been proposed as early as 1962 (Freudenthal, 1962), but thus far could not be confirmed. Still, genetic evidence clearly point towards sexual reproduction in this dinoflagellate genus (Baillie et al., 2000; Belda-Baillie et al., 1999; LaJeunesse, 2001; LaJeunesse, 2005). If *Symbiodinium* is reproducing sexually, genetic recombination can be assumed to be much higher than in strictly asexual organisms, with important implications for the fitness and adaptability of *Symbiodinium* and the resilience of corals to stressful conditions, such as coral bleaching events (Baker, 2003; Putnam et al., 2012). The fact that reactive oxygen species (ROS) play an important role in both the phenomenon of coral bleaching (Fridovich, 1998; Hoegh-Guldberg, 1999; Weis, 2008) and in triggering the sexual life cycle in other algae (Nedelcu et al., 2003, 2004; Nedelcu, 2005) also raises important questions on potential links between these two processes.

A major aim of the present work was to investigate the sexual life cycle of *Symbiodinium* spp. and thus to start closing this critical gap in our understanding of *Symbiodinium* spp. biology. The first hypothesis of the present work is that *Symbiodinium* spp. must have a sexual reproduction stage in its life cycle if it has such a high genetic diversity. In order to identify gametes, to

determine mating-type affiliations and the formation of zygotes, crossing experiments were planned within different clades and between different clonal strains. One of the first goals to enable these experiments was to establish a culture collection by isolating single cells from cnidarian hosts and by attaining additional cultures from other collections. Genotyping of the cultures based on different genetic markers was intended to help in the identification of clades and ITS-types and thus to chose promising candidate strains for experimentation. Another important goal was the identification of known vegetative life cycle stages in order to build a basis for the discovery of new and potentially sex-related life cycle stages.

The fact that the free-living life cycle of *Symbiodinium* spp. is still poorly studied (Andras et al., 2011; Takabayashi et al., 2012) also explains why direct cell-cell interactions, which are commonly found in obligate free-living dinoflagellate species (Uchida, 1991, 2001; Uchida et al., 1996, 1995, 1999) have not been described yet for *Symbiodinium* spp. This entirely unexplored aspect of *Symbiodinium* ecology may have important implications for the growth of competing strains in the environment and for the outcome of host colonization by competing symbiotic strains.

The second major aim of the present work was to identify interactions between *Symbiodinium* spp. strains and to start characterizing the effects these interactions have on growth characteristics. The second hypothesis of the present work is that *Symbiodinium* spp. must display competition against other microalgae if it has a free-living life cycle stage. To identify cell-cell interactions, it was intended to perform further crossing experiments, with a focus on the crossing of strains from different clade and ITS-type identities and to document the interactions using light- and epifluorescence microscopy. To characterize the effects of strain interactions, an important goal was to establish a suitable method for the identification and separate quantification of interacting strains in co-culture.

# 2. Materials and methods

## 2.1. Culture collection

The *Symbiodinium* spp. cultures used in this study had two main sources. Approximately 150 cultures were directly isolated from the coral *Sinularia* flexibilis and the tropical anemone, *Aiptasia pallida* with glass pasteur pipettes. Further 50 cultures were provided by Mark Warner's lab. A more detailed overview of 31 of these cultures is described in table 27.

## 2.2. DNA Extraction

The extraction of DNA for further molecular analyzes was adapted from Frommlet et al. (2008), and served two purposes: being suitable for downstream PCR amplification of rDNA and for the amplification of the microsatellite B7Sym15 and its flanking region. The single cell or the cell suspension was vortexed with sterile glass beads in order to disrupt the cells and to expose its DNA content

**Table 1 –** Protocol for the DNA extraction

| |
|---|
| 1 – Centrifuge 100 µL of each culture at 1200g for 2 minutes; |
| 2 – Discard the supernatant; |
| 3 – Add 10 µL of ultrapure water; |
| 4 – Add 10 to 15 glass beads to each tube and vortex (VWR Analog Mixer) for 1 minute. Sample is prepared for PCR amplification. |

## 2.3. DNA Amplification

PCR reactions were performed in 50 µL volumes and on a termocycler HX0128-00208 Eppendorf. The following reagents were acquired from VWR and used according to table 2:

**Table 2 –** MasterMix used in the PCR reactions

| Master Mix | |
|---|---|
| Taq Polymerase | 0,4 µL |
| dNTP's | 1,0 µL |
| Primers (F/R) for ITS | 2,5 µL |
| 10X buffer | 5,0 µL |
| MilliQ water | 37,6 µL |
| Template | 1,0 µL |
| Total | 50 µL |

## 2.4. Polymerase Chain Reaction (PCR)

The DNA regions 18S rDNA; ITS1-5.8-ITS2 and 28S rDNA were targeted for genetic screening of the cultures. These regions were amplified by polymerase chain reaction, which is a technique based on a repetitive series of cycles (usually between 20 and 40) which, in turn, consist of three critical steps:

Denaturing step: the double stranded DNA is denatured by increasing the temperature to 95ºC. At this temperature, the hydrogen bonds of the double stranded DNA are broken, thus originating single DNA strands.

Annealing step: the temperature is lowered to 50-65ºC for 20-40 seconds in order to allow the annealing of the primers to the single-stranded DNA template. The annealing of the primers occur at a lower temperature than the melting temperature of the of the oligonucleotide primers.

Elongation step: after the annealing of the primers, the Taq DNA polymerase recognizes the primer-template sequences and binds to them. Taq polymerase has its optimum temperature between 75-80ºC and during this step,the DNA

polymerase synthesizes a new strand of DNA, which is complementary to the DNA template strand, by adding dNTPs in 5' to 3' direction. The newly amplified double-stranded DNA strands will be used as the template for the next PCR cycles.

For more details on the protocols, see annexes.

**Table 3** – PCR profile for the amplification of the general eukaryotic 18S rDNA region, adapted from Rowan et al. (1991)

| Molecule | Primer | Step | Temperature (ºC) | Time (min) |
|---|---|---|---|---|
| 18S rDNA (~1800bp) (General 18S rDNA Eukaryotic primer) | SS5 SS3 | 1 | 94 | 2:30 |
| | | 2 | 56 | 1:00 |
| | | 3 | 72 | 2:00 |
| | | 4 | 94 | 1:00 |
| | | 5 | 56 | 1:00 |
| | | 6 | 72 | 2:30 |
| | | | 29x repetitions from step 4 | |
| | | 7 | 72 | 8:00 |
| | | 8 | 4 | Hold ∞ |

**Table 4 –** PCR profile for the amplification of the 18S rDNA region, adapted from Santos et al. (2003)

| Molecule | Primer | Step | Temperature (ºC) | Time (min) |
|---|---|---|---|---|
| 18S rDNA (~750bp) | SS5 SSE21.6 | 1 | 94 | 2:30 |
| | | 2 | 56 | 1:00 |
| | | 3 | 72 | 2:00 |
| | | 4 | 94 | 1:00 |
| | | 5 | 56 | 1:00 |
| | | 6 | 72 | 2:30 |
| | | | 29x repetitions from step 4 | |
| | | 7 | 72 | 8:00 |
| | | 8 | 4 | Hold ∞ |

**Table 5 –** PCR profile for the amplification of the ITS rDNA region, adapted from Santos et al. (2001)

| Molecule | Primer | Step | Temperature (ºC) | Time (min) |
|---|---|---|---|---|
| 18S rDNA (~750bp) | ZITSUP ZITSDN | 1 | 94 | 2:00 |
| | | 2 | 94 | 0:30 |
| | | 3 | 60 | 0:30 |
| | | 4 | 72 | 0:45 |
| | | | 35x repetitions from step 2 | |
| | | 6 | 72 | 5:00 |
| | | 8 | 4 | Hold ∞ |

**Table 6 –**PCR profile for the amplification of the 28S rDNA region, adapted from T.P. Wilcox (1998b)

| Molecule | Primer | Step | Temperature (ºC) | Time (min) |
|---|---|---|---|---|
| 28S rDNA (~900bp) | ls 1.5 ls 1.3 | 1 | 94 | 2:00 |
| | | 2 | 94 | 0:15 |
| | | 3 | 52 | 0:20 |
| | | 4 | 72 | 0:45 |
| | | | 29x repetitions from step 2 | |
| | | 6 | 72 | 7:00 |
| | | 8 | 4 | Hold ∞ |

## 2.5. DNA quantification and quality assay

Prior to the agarose gel electrophoresis, the quality of the amplicons was assessed spectrophotometrically using an ND-1000 spectophotometer (NanoDropTM, Wilmington, DE, USA). This technique is based in the analyses of UV absortion by the nucleotides, which provides a simple and accurate estimation of the concentration of nucleic acids in a sample.

Agarose is a polymer which is extracted from seaweeds and is commercially sold as a white powder. The powder is usually melted in a buffer (e.g. TAE 1X) and after cooling, it forms a gel by hydrogen bonding. Depending

on the concentration of the agarose in the gel, the bigger or smaller will be the size of the pores of the matrix formed by the gel. Despite the several possible techniques, the widely used “submarine” gel system was used. The gel was cooled in a supporting plate which was submerged in a suitable electrophoresis buffer. The samples were loaded and the electric field was applied. This principle has its basis on the negatively charged DNA at neutral pH, which migrates from the negative pole towards the positive pole. According to the percentage of agarose in the gel, the molecules will be separated accordingly to their size (in kb). Shorter molecules of DNA will migrate faster through the matrix, whereas larger molecules will move slower. Gel electrophoresis (2% agarose; 1-2 µL ethidium bromide)

### 2.6. Purification of PCR products

The amplified fragments were purified utilizing QIAquickTM PCR purification kit (Qiagen). This protocol is based on the basis of reversible adsorption of nucleic acids to silica gel at high pH and in the presence of high concentrations of chaotropic salts (substances that disrupt the hydrogen bonds in water, interfere with the structure of the DNA and decrease its solubility in water).

**Table 7 –** Protocol for the purification of PCR products with a QIAquickTM PCR purification kit (Qiagen)

| **Notes before starting:** |
| --- |
| • Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).<br>• All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.<br>• Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots. |

**Protocol:**

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in a provided 2 ml collection tube or into a vacuum manifold.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60s or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column centrifuge for 30–60s or apply vacuum. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

## 2.7. Molecular Discrimination of Phylotypes

The purified amplicons were sequenced by a commercial provider (StabVida). The sequence data were processed using the software BioEdit and blasted in GenBank in order to determine the phylotype of samples.

## 2.8. Establishing of Monoclonal Cultures

The isolation of monoclonal cultures of *Symbiodinium* spp. was performed with tissue samples from the coral *Sinularia* flexibilis and anemone *Aiptasia pallida*. The picked cells were inoculated in F/2 medium, which is a commonly used medium to grow dinoflagellates (Guillard et al., 1962; Kinzie et al., 2001; Santos et al., 2001)

**Table 8 –** Protocol for the establishment of monoclonal cultures

1- Collect fragments of the tissue of *Sinularia* flexibilis and *Aiptasia pallida*;
2- Introduce the tissue samples in 15 mL Falcon tubes and wash 3 times with fresh F/2 medium, in order to remove free-living algal cells from the aquarium system.
3- Homogenise the samples with an Ultra Turrax (IKA Labortechnik U200S control), to release the symbionts from the cnidarian tissue;
4- Pick single cells under an inverted microscope (Leitz Laborvert FS), using thinned-out glass capillary or 1-10 µl micropipettes (Eppendorf Research Plus);
5- Make successive dilutions of the crude extracts, using F/2 medium, until the concentration of symbiont cells allows single cell picking;
6- Pick single cells;
7- Inoculate each cell in 200 µL of F/2 medium, using a 96 well plate (Sarsted flat bottom suspension cells). The outer 36 should be filled with ultrapure water in order to prevent evaporation of the medium.
8- When cells reach approximately 80% of confluence, transfer 0.2 mL from the mother culture and 1.8 mL of fresh F/2 medium to 24 well plates.

## 2.9. F/2 medium

Stock solutions of the F/2 medium were prepared so that when one needed fresh F/2 medium, it was only add the stock solution to one liter of filtered and autoclaved seawater. The F/2 medium used during the course of the work was made without the silicate stock solution, once that the dinoflagellates do not need so many silicates as diatoms do. Thus, the

formation of silicate precipitates could represents a danger for the flow cytometer, once they could clog the equipment.

**Table 9 –** Recipe for F/2 medium. Adapted from Guillard & Ryther, 1962

| F/2 medium | | | |
|---|---|---|---|
| **Stocks** | | | |
| | **(1)** | **Trace elements (chelated)** | **Per liter** |
| | | Na2EDTA | 4.6 g |
| | | FeCl3.6H2O | 3.15 g |
| | | CuSO4.5H2O | 0.01 g |
| | | ZnSO4.7H2O | 0.022 g |
| | | CoCl2.6H2O . | 0.01 g |
| | | MnCl2.4H2O | 0.18 g |
| | | Na2MoO4.2H2O | 0.006 g |
| | **(2)** | **Viamin mix** | |
| | | Cyanocobalamin (Vitamin B12) | 0.0005 g |
| | | Thiamine HCL (Vitamin B1) | 0.1 g |
| | | Biotin | 0.0005 g |
| **Medium** | | | **Per liter** |
| | | NaNo3 | 0.075 g |
| | | NaH2PO4.2H2O | 0.00565 g |
| | | Trace elements stock solution (1) | 1.0 mL |
| | | Vitamin mix stock solution (2) | 1.0 mL |

## 2.10. Culture maintenance

Usually, cell cultures will suffer a number of doublings until the culture reaches the stationary phase. Cell cultures of *Symbiodinium* spp. have been cultures for decades without reaching a senescence which is determined by a number of specific factors of the cell's cycle regulation (Freshney, 2006). When a cell line is sub-cultured, it will grow again until reaching the same cell density it had before the subculture, which is no more than plotting a growth curve from a sample of the cell line taken at specific intervals along the growth cycle. This shows that cells can stop growing, entering a period of latency after reseeding – the *lag period* (Freshney, 2006). Afterwards, a phase of exponential growth – *log phase* – occurs, in which the cell population doubles over a specific period (the doubling time) which is characteristic of each cell line. When the cell

population is crowded, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle. Subsequently, the cells enter the *plateau* or *stationary phase*, in which there is almost no culture growth. Cells may be subcultured from plateau, though it is preferable to do it before they reach this phase, in the top end of the log phase, in which the growth fraction is higher and the recovery time (lag period) shorter (Freshney, 2006).

**Table 10 –** Protocol for subculturing of the *Symbiodinium* spp. cultures

| |
|---|
| 1 – After four weeks of incubation, cultures are subcultured into new 24 well plates; |
| 2 – Prepare fresh F/2 medium and store in the culture incubator for 1 hour to match the temperature of the cultures; |
| 3 – Add 1.8 mL of F/2 medium and 200 µL of mother culture to each well; |
| 4 – All the steps must be done under aseptic conditions; |
| 5 – All the cultures of the collection are grown under a 12:12 photoperiod (150 µmol photons.$m^{-2.s-1}$) at 26ºC (Biotronette plant growth chamber). |

## 2.11. Bright Field Microscopy

In biological studies, the images can be measured either by common optical methods such as optical microscopy or by more advanced methods that provides additional physical or chemical information about the objects. Studies of biological structures and processes on both fixed and live specimens have advanced light microscopy into an indispensable tool for cell and molecular biologists (Keller et al., 2006). The light microscope creates a magnified, detailed image of seemingly invisible objects or specimens, based on the principles of transmission, absorption, diffraction, and refraction of light waves. The various types of microscopes produce images of objects employing different strategies. In all instances (e.g., bright field, phase contrast, and fluorescence), the production of a clear and informative image is dependent on the magnification of the object, its contrast with respect to its internal or external surroundings, and the ability to resolve structural details (Keller et al., 2006). The visibility of the magnified object depends on contrast and resolution. In

general, the contrast or differences in light intensity between an object and its background or surroundings render the object distinct. For colorless specimens, as is the case for most biological material, contrast is achieved in various ways. The object itself or selected portions of it may be stained, thus reducing the amplitude of certain light waves passing through the stained areas. However, this usually requires the killing or fixation and staining of cells. Such stained specimens are typically observed using bright-field microscopy (Keller et al., 2006).

### 2.12. Epifluorescence microscopy

Fluorescence microscopy enables the confirmation of the positive binding of the dyes to the nucleic acids, prior to the flow cytometric analyses of the cultures. Propidium iodide (PI) binds between the nucleotide pairs of guanine and cytosine. It stains not only the DNA but the RNA as well (Suzuki et al., 1997), thus, RNAse digestion was performed in parallel with the DNA staining in the dark for 2 hours at room temperature (see annexes for more details on the protocol). PI stained cells show a red nucleus under the green-yellow (568nm) or blue (488nm) laser excitation (e.g. Fig 13).

### 2.13. Flow Cytometry

Flow cytometry allows grouping individuals simultaneously by size, chemical composition, and/or metabolic state. Once it allows measuring a large number of individuals in a short amount of time, it ultimately leads to the acquisition of new insights into ecosystems structure and dynamics. Such measurements are possible on individual cells in the 1-150 µm size range. Flow cytometry is a general technique for the rapid measurement of particles in a moving fluid and information is obtained through their optical properties. The fluorescence or light scatter is detected by photomultiplier tubes; forward angle light scatter, which is some function of cell size, is detected by a photodiode. The use of lasers permits selection of specific coherent excitation wavelengths at high intensity which maximizes fluorescence emission (Kron et al., 2007; Rahman, 2004). With the aid of laser-based FCM and cell sorting, pigment

autofluorescence, stain-induced fluorescence, and light scatter are used as probes to quantify and sort subpopulations of phytoplankton cultures and natural populations. This technique, when combined with biochemical selective and immunofluorescence technologies, makes possible simultaneous measurement of multiple properties, as chlorophyll, protein, DNA, light scatter, of individual cells and particles (Kron et al., 2007; Rahman, 2004).

**Table 11** – Preparation of the samples for flow cytometry

| |
|---|
| **1 -** Fix the culture with 1% paraformaldehyde for 10 minutes; |
| **2 -** Wash the culture with 1% PBS and remove it by centrifugation at 1200g for 10 minutes (Eppendorf 5810 R); |
| **3 -** Add 5mL of Methanol (chlorophyll extraction) at 4ºC, resuspending the culture and store it for 12 hours at 4ºC (GPR Rectapur VWR); |
| **4 -** Wash the cells twice as described in step 2; |
| **5 -** Add 900 µL of PBS and 100 µL of staining solution (50 µL of Propidium Iodide plus and 50 µL of RNaseA); |
| **6 –** Store the culture in the dark during 2 hours before analysis. |

## 2.14. 4% paraformaldehyde

**Table 12** – Recipe for the preparation of 4% paraformaldehyde

| **4% paraformaldehyde** |
|---|
| 1 - Mix 4g of paraformaldehyde (95%) in 80 mL of ultrapure water; |
| 2 - Heat solution to 56ºC and keep stirring; |
| 3 - Adjust pH to 11; |
| 4 - Once the solution is clear, let it cool down at room temperature; |
| 5 - Adjust pH to 7.4 and the volume to 90 mL with ultrapure water; |
| 6 - Add 10 mL of 10x PBS to a final concentration of 4% paraformaldehyde in 1% PBS. |
| 7 - Sterilize the solution using a 0.22 µm filter and store at 4ºC. |

**Table 13 -** Recipe for the preparation of 10X Phosphate Buffer Saline 1L

| **10x Phosphate Buffered Saline 1L** |
|---|
| 80g NaCl |
| 2g KCl |
| 14.4g $Na_2HPO_4$ |
| 2.4g $KH_2PO_4$ |
| Adjust pH to 7.4 |

## 2.15. Crossing Experiments

For the search of study of both sexual reproduction and strain interactions in the dinoflagellate *Symbiodinium* spp., several crossing experiments were performed. A crossing experiment consists of the mixture of two monoclonal cultures in order to study the interactions between the cultures.

**Table 14** – Protocol for crossing experiments

| |
|---|
| 1 - Mix two different cultures in 24 well tissue culture plates or in conventional microscopy slides, sealing with vaseline to prevent evaporation; |
| 2 – Utilize a compound microscope (Leitz Laborlux S), in the case of cover slides, or an inverted microscope (Leitz Labover FS), in the case of 24 well tissue culture plates; |
| 3 - For both microscopes, the use of a digital camera is a must in order to document the outcome of crossing experiments. |

## 2.16. Deconvolution of gathered data by flow cytometric analyses

In the present study, flow cytometry was used to assess individual cell characteristics on a Coulter EPICS XL (Coulter Electronics, Hialeah, FL, USA)

for a large number of cells. The analysis of flow cytometric data presented some challenges, as the presence of overlapping distributions, masking in part the subpopulations of cells. The deconvolution of this data in the software Plot (v1.997) allowed estimating the populations of each strain in the crossing experiments, including the overlapping populations. Based on the characteristic DNA content of each strain grown in isolation (figure 25) as a comparison, the percentage of each strain during the course of the crossing experiments was estimated for each time point.

# 3. Results

### 3.1. Genetic characterization of cultures isolated from *Aiptasia pallida* and *Sinularia* flexibilis based on rDNA sequencing

For an initial genetic screening of the isolated cultures from *Aiptasia pallida* and *Sinularia* flexibilis, four cultures each were randomly selected. The DNA from these eight cultures was extracted and a partial region of the small-subunit ribosomal RNA genes (SSU rDNA or 18S), the entire Internal Transcribed Spacer (ITS1-5.8S-ITS2 or ITS) and a partial region of the large-subunit ribosomal RNA genes (LSU rDNAor 28S) were amplified and analysed by gel electrophoresis. The PCR reactions with the SSU rDNA primers did not produce any amplicons. The PCRs targeting the other two rDNA regions yielded amplicons of expected size for all eight cultures, indicating that the isolated cultures were strains of *Symbiodinium* spp. (Figure 9).

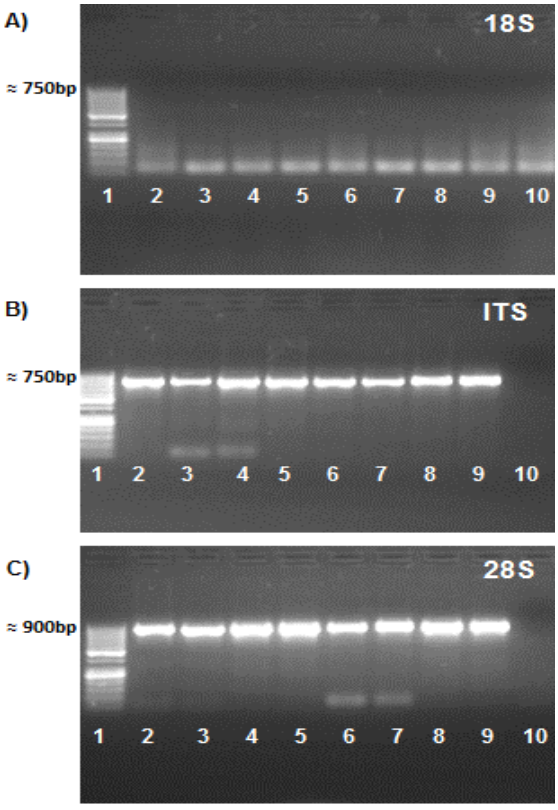


**Figure 9 -** Agarose gel electrophoresis of the 18S (A), ITS1-5.8S-ITS2 (B) and 28S (C) rDNA, showing the expected amplicon sizes of ≈750bp, 750bp and 900bp, respectively. Lane 1 – 50bp DNA ladder; 2, 3, 4 and 5 – clonal cultures isolated from *Aiptasia pallida*; 6, 7, 8 and 9 - clonal cultures isolated from *Sinularia* flexibilis; 10 – negative control. Gel = 2% agarose in 1xTAE buffer, stained with ethidium bromide.

To verify the results obtained by gel electrophoresis and to attain more detailed information about the identities of the cultures, two randomly selected 28S amplicons and all eight ITS amplicons were purified and sequenced. The two 28S sequences were identical and blasting against GenBank resulted in exact matches with a clade B sequence in the database. The eight ITS sequences were also identical among themselves and an exact match was found in GenBank with a type B1 sequence (Table 15). Together these results established that all eight cultures were strains of *Symbiodinium* type B1 and suggested a dominance of type B1 strains in the isolates of both cnidarian hosts.

**Table 15** - Blasting results of the two 28S and the eight ITS sequences against GenBank. n/r – not resolvable.

| Samples | Molecular marker | Clade | Type | Accession number (NCBI) |
|---|---|---|---|---|
| SPI. (1 and 2) | 28S | B | n/r | U63484.1 |
| 24R.(1 to 8) | ITS | B | B1 | EU074864.1 |

### 3.2. Identification of known vegetative life cycle stages of *Symbiodinium* spp.

To establish a basis for the identification of novel life cycle stages with potential involvement in sexual reproduction, known vegetative life cycle stages of *Symbiodinium* spp. had to be identified first. With the exception of the aplanospore (which previously was found only on solid medium) and the binary fission of the vegetative cells (which only occurs *in symbio*), all described vegetative life cycle stages of *Symbiodinium* spp. in culture could be identified by light- and epifluorescence microscopy.

### 3.3. Vegetative cells (cysts)

Vegetative cells were identified based on the lack of motility and their round to oval shape (Fig. 10). Vegetative cells were found in all studied cultures, including clade A, B, C and F cultures. The relative abundance of vegetative cells compared to motile zoospores changed diurnally. During the dark period, cultures only contained vegetative cells. With the onset of the light period, vegetative cells produced motile zoospores (see below for details) and towards the end of the light period the zoospores settled down and again formed vegetative cells.

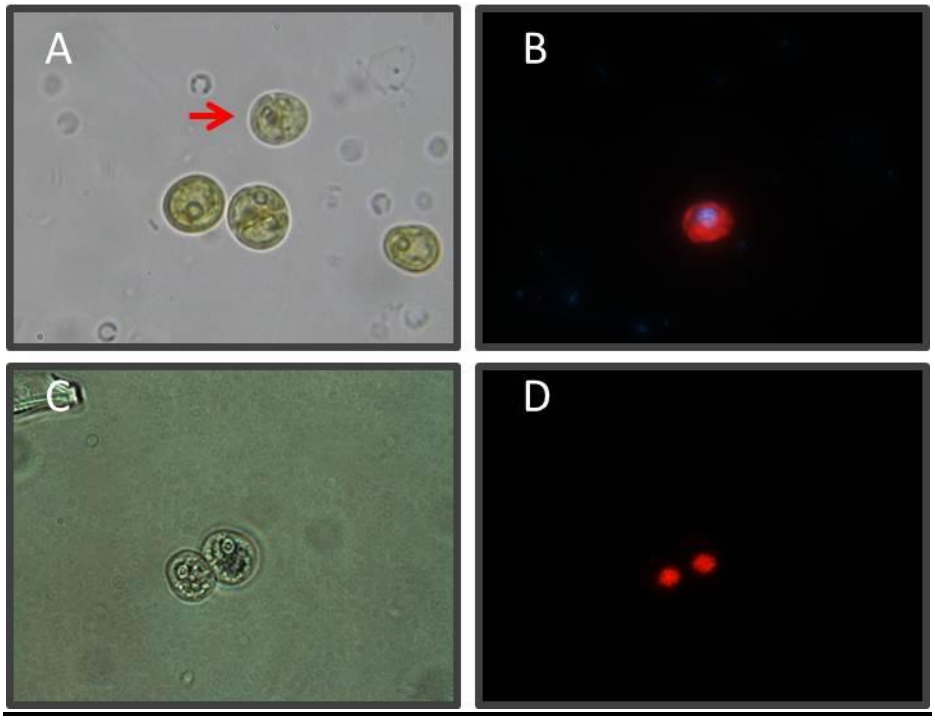


**Figure 10 -** Vegetative cells (cysts) of *Symbiodinium* type B1. A - Live vegetative cells under the light microscope, B - DAPI live staining of a vegetative cell under the epifluorescence microscope (red – chlorophyll fluorescence, blue – fluorescence of the DAPI stained nucleus), C – Two vegetative cells fixed with 1% paraformaldehyde for flow cytometric analyses, D – the same cells as in C, stained with propidium iodide (red fluorescence of the nucleus stained with propidium iodide)

### 3.4. Zoospores and zoosporangia

The zoospores of *Symbiodinium* spp. were identified based on their shape and the motility of this cell type. In agreement with the literature, zoospore formation was controlled by the light-dark period. During the dark period, zoospores were completely absent from the cultures. The gymnodinoid zoospores emerged from zoosporangia starting with the onset and continuing to approximately half (6 h) of the light period. Qualitative microscopic observations further suggested that peak abundances of zoospores varied between clades, clade F strains producing noticeably lower zoospore numbers than clade A, B, and C strains, and showed a negative correlation between zoospore abundance and late growth stages of the cultures. Zoosporangia were found to give origin to one (Fig. 11 E), two (Fig. 11 C) and four motile zoospores (Fig. 11 A). Mitotically dividing cells were observed frequently and in all studied strains. Both nucleic acid dyes, propidium iodide and DAPI greatly facilitated the identification of dividing cells (Fig. 10 B & D, respectively). A number of live dividing cells were followed throughout the entire division process and all resulted in the formation of zoospores. Most of the cells in division produced two zoospores. In a single documented case, a large cell produced four zoospores (flagella were visible when zoospores emerged) but shortly after, the emerged cells settled down (Fig. 11 A). The formation of non-motile vegetative daughter cells directly from their mother cells, as described in the literature, was never observed.

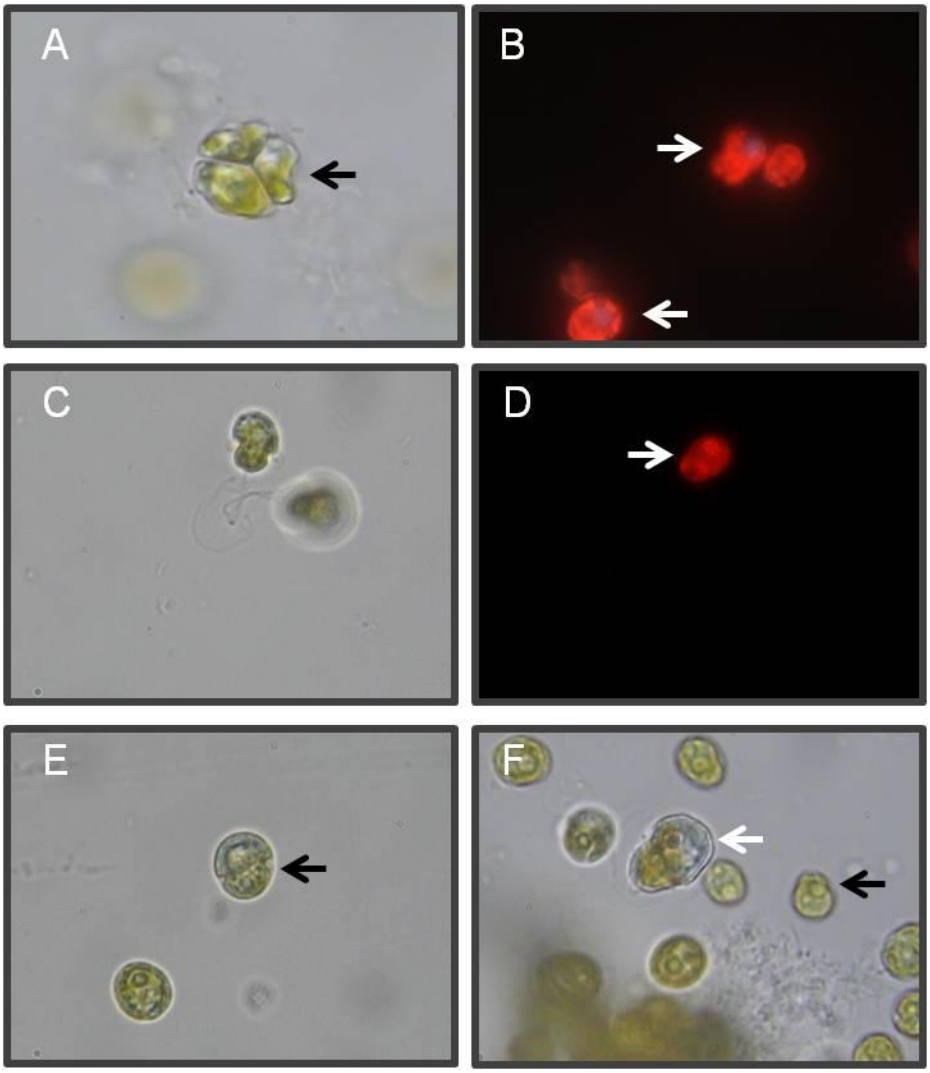


**Figure 11 -** A – Tetrad cell under the light microscope (after the rupture of the cell wall, four zoospores emerged), B - DAPI live staining of two zoospores under the epifluorescence microscope (in red – chlorophyll fluorescence, in blue – blue fluorescence of the nucleus stained with DAPI), C – A couple of zoospores emerging from a zoosporangium leaving behind the empty cell wall, D – Zoospore stained with propidium iodide (see detail of the nucleus red fluorescence, located in its epicone), E – Zoosporangium with a single developing zoospore (see the detail of the groove where the transversal flagellum is located), F – A particularly big zoospore (white arrow) and a regular sized zoospore (black arrow)

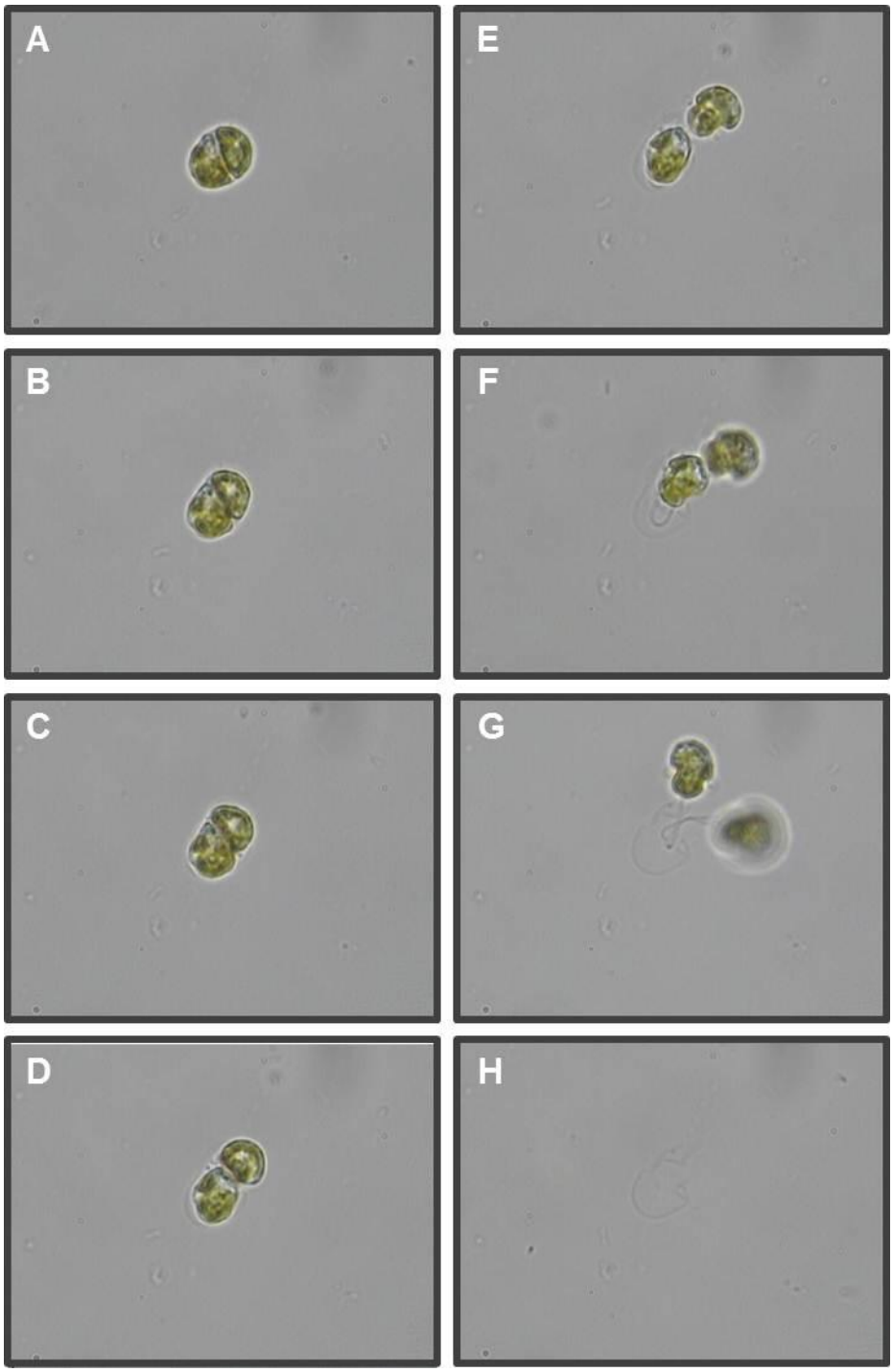


**Figure 12 -** Time series of two zoospores emerging from a zoosporangium. A-G – Different stages of the zoosporangium,; H – Empty cyst after zoospores. Total length of the video from A (initial rupture of the cell wall of the zoosporangium) to H (final detachment of both zoospores) – 105 seconds.

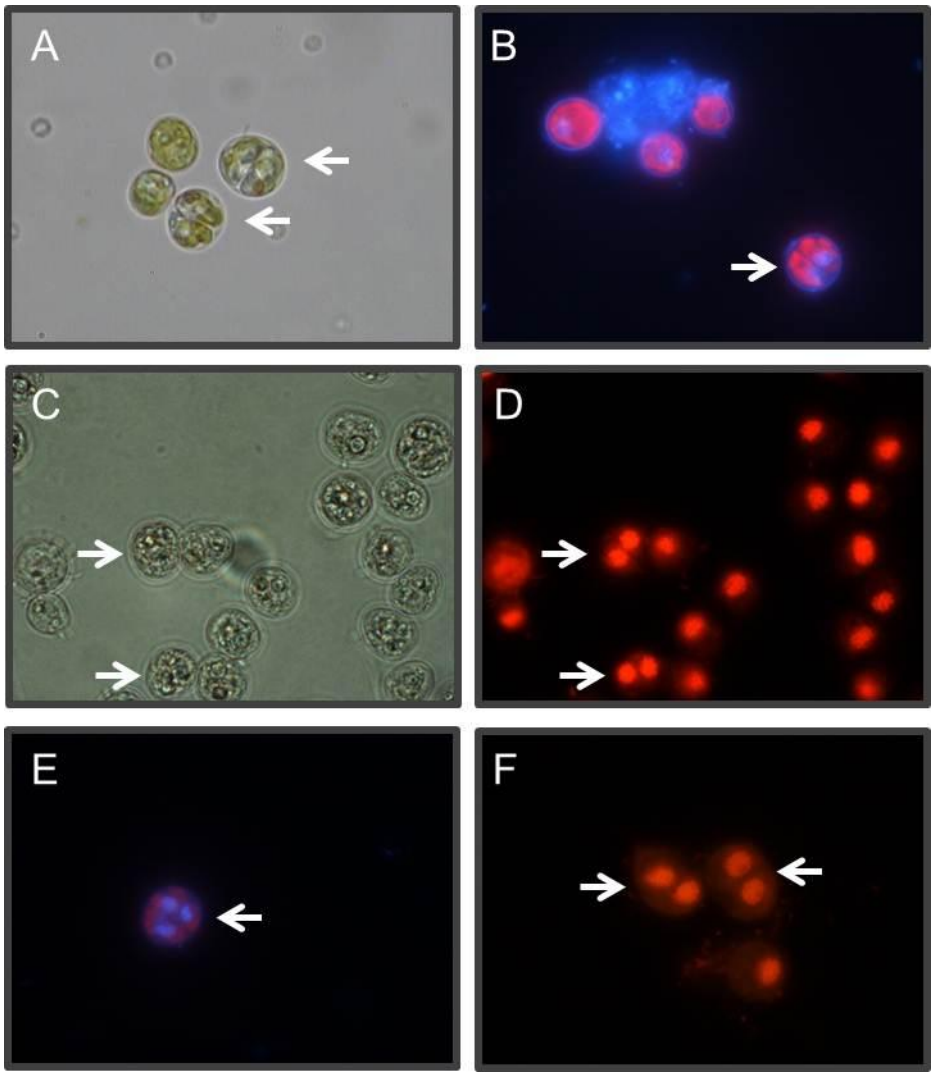


**Figure 13 -** Vegetative cell division in *Symbiodinium*. A – Two live zoosporangia, each containing two developing zoospores; B - DAPI live staining of a dividing cell under the epifluorescence microscope (red – chlorophyll fluorescence, blue – fluorescence of the nucleus stained with DAPI); C – Light microscopy image of cells fixed with 1% paraformaldehyde and stained with propidium iodide for later flow cytometric analyses. Notice that the dividing cells, pointed out by arrows, are morphologically very similar to the other cells; D – The same cells as in C under the epifluorescence microscope. The nuclear staining revealed clearly which cells were dividing; E – Vegetative cell dividing into four daughter cells (three cells in focal plane and one cell below focal plane); F – Doublets of dividing cells, stained with PI.

### 3.5. Study of sexual reproduction in type B1 cultures

Light microscopic observations lead to the discovery of round, cell-like bodies in most of the isolates from *A. pallida* and *S. flexibilis*. The term “cell-like bodies” is used here because later tests (see below) could not conclusively establish whether they were actual cells, or not. The bodies displayed internal granulation and structure and appeared translucent without any apparent pigmentation (Figure 6). In one culture, the size of the cell-like bodies and that of normal vegetative cells was measured. Compared to the size of the vegetative cells with 7.1 µm, the cell-like bodies, with 2.1 µm, were very small (Fig. 14). Their round shape and their size were two features that were in agreement with the single previous description of putative gametes of *Symbiodinium* spp. To test whether these bodies could indeed be gametes, crossing experiments were conducted between different strains from *A. pallida* and *S. flexibilis* culture collection. These experiments did not result in any interaction between the bodies (attraction or fusion) that would have indicated them to be gametes (results not shown). In the course of these crossing experiments, other cell types (zoospores and vegetative cells) were also studied for signs of interaction. Again, no clear evidence for any sexual life cycle process could be found.

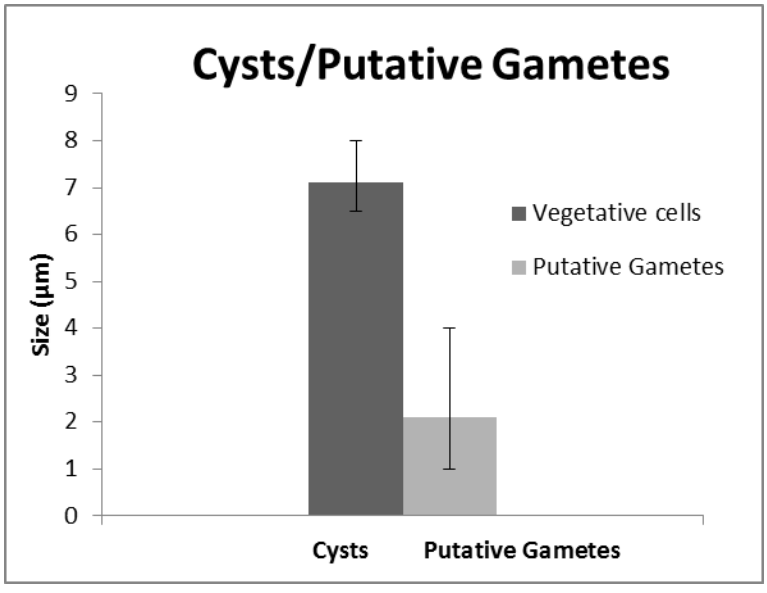


**Figure 14 -** Cell size chart of *Symbiodinium* spp. cysts, putative gametes and respective error bars.

Parallel to the crossing experiments, other methods were used to clarify the identity of the cell-like bodies. One approach was to separate the normal vegetative cells from the much smaller bodies by filtration and then to

genetically characterize the bodies based on rDNA amplification and sequencing. Filtration resulted in two distinct sample fractions: one with high concentration of vegetative cysts of *Symbiodinium* spp. with small amounts of cell-like bodies and another one with high concentrations of the cell-like bodies without any visible contamination with vegetative cells. PCR amplification of the filtrates with ITS (ZITSUP and ZITSDN) and 28S (ls 1.5 and ls 1.3) primers did not yield amplicons for the cell-like bodies (data not shown).

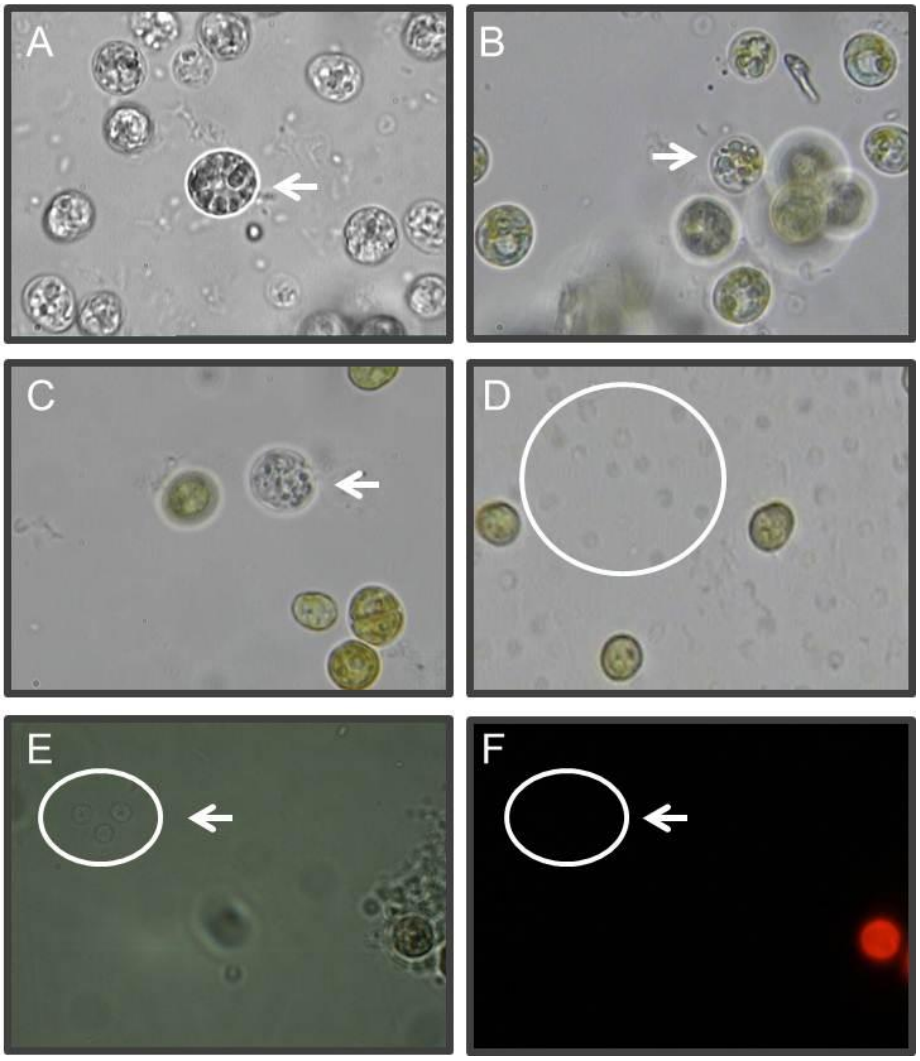


**Figure 15 -** Putative gametes and its precursor cells. A-C – Live culture of *Symbiodinium* sp. isolated from *Aiptasia pallida* (likely a type B1). The arrow highlights the putative gamete precursor cell; D/E – Putative gametes of *Symbiodinium* spp.; E/F - Light microscopy image of three putative gametes, fixed with 1% paraformaldehyde and stained with propidium iodide for later flow cytometric analyses. D – The same cells as in E under the epifluorescence microscope, showing the absence of nucleic staining in the putative gametes.

In a similar approach, single cell-like bodies were picked for PCRs with the *Symbiodinium*-specific primers SS5 and SS3Z (forward and reverse primers for the amplification of the ITS region); ZITSUP and ZITSDN (forward and reverse primers for the amplification of the ITS region). The PCRs with ITS primers were not conclusive due to contamination problems and lack of amplification in positive controls but PCRs with 18S primers gave clear results (Figure 7). The 18S PCRs consistently yielded amplicons from positive controls but did not yield amplicons from the cell-like bodies. This lack of amplification with *Symbiodinium*-specific primers gave further evidence that the tested cell-like bodies were not gametes of *Symbiodinium* spp., nor any other *Symbiodinium* spp. cell type for that matter.

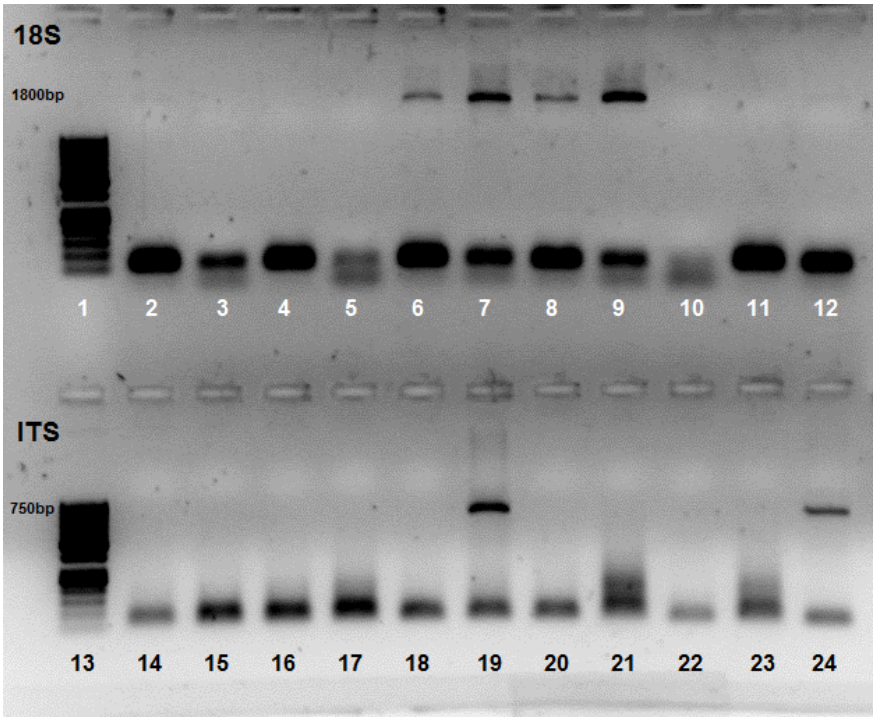


**Figure 16 -** Agarose gel electrophoresis of single cell PCRs using the 18S primers: SS5 and SS3Z (top) and the ITS primers: ZITSUP and ZITSDN (bottom). Lanes 1 and 13 – 50bp DNA ladder; Lanes 2-5 and 14-17 – Single cells of putative gametes, not yielding amplicons; Lanes 6-9 and 18-21 – Single vegetative cells (positive controls), resulting in 18S amplicons of expected size (≈1800bp) for all 4 cells and in ITS amplification for only a single cell (lane 19, ≈750bp); Lanes 10-12 and 22-24 – Negative controls, showing expected results for the 18S PCR reactions but yielding an amplicon for the ITS reactions (lane 24), indicating contamination.

*Symbiodinium* spp. is haploid and thus both vegetative cells and gametes should have the same DNA content. In another attempt to find out more about the small cell-like bodies, propidium iodide staining was used to investigate their

nuclear DNA content by epifluorescence microscopy and flow cytometry. Whereas the applied protocol was effective in staining the DNA of vegetative cells (e.g. Figs. 10 D and 13F), the bodies showed no nuclear staining (Fig. 14 E & F). Since the bodies displayed no visible nuclear staining, it was not surprising that flow cytometry also failed in detecting the population of small cell-like bodies (results not shown).

### 3.6. Microsatellite analysis of the *A. pallida* and *S. flexibilis* culture collection

The apparent lack of sexual reproduction between cultures from *A. pallida* and *S. flexibilis* and the apparent dominance of a single ITS-type raised the question of whether the different cultures could have been clonal in origin and thus potentially incompatible for sexual reproduction. To investigate this possibility, another fifty of these cultures were randomly selected for analysis of the microsatellite locus B7Sym15 and its flanking region by PCR amplification and sequencing (Figs. 17 & 18, respectively). The flanking region of all fifty cultures were identical among themselves and identical to six reference cultures of symbiotic B1 strains from Mark Warner`s culture collection. Two potentially free-living strains of type B1 *Symbiodinium* sp. could be distinguished from all other cultures based on differences in the MS flanking region. The microsatellite locus B7Sym15 itself, with its two dinucleotide repeats $(GT)_n$ and $(AC)_n$, was also identical in all fifty cultures, indicating that the screened cultures were potentially of clonal origin. Identical reference cultures were NEW AIP and OLD AIP, both isolated from *A. pallida* by the Warner lab. The potentially free-living cultures 146 and 147 also shared this microsatellite repeat signature but, as mentioned above, were distinguishable based on differences in the MS flanking region. The remaining four symbiotic B1 reference cultures PK 702, PK 704, and M. cap. grouped together, whereas culture 74 had a distinct MS genotype. Thus, the tested 56 symbiotic B1 cultures fell into three groups of distinct MS genotypes.

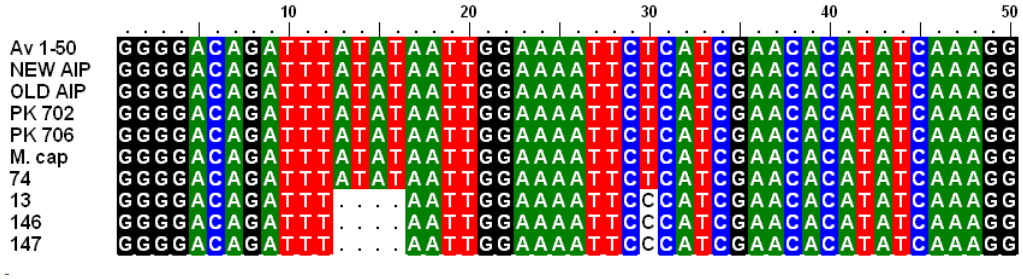


**Figure 17 -** Sequences alignment of the Microsatellite locus B7Sym15 (MS) flanking region. Av1-50 = consensus sequence of 50 type B1 strains isolated from *Aiptasia pallida* and *Sinularia* flexibilis. NEW AIP - 74 = MS flanking region of symbiotic B1 strains. 13 – 147 = MS flanking region of potentially free-living B1 strains.

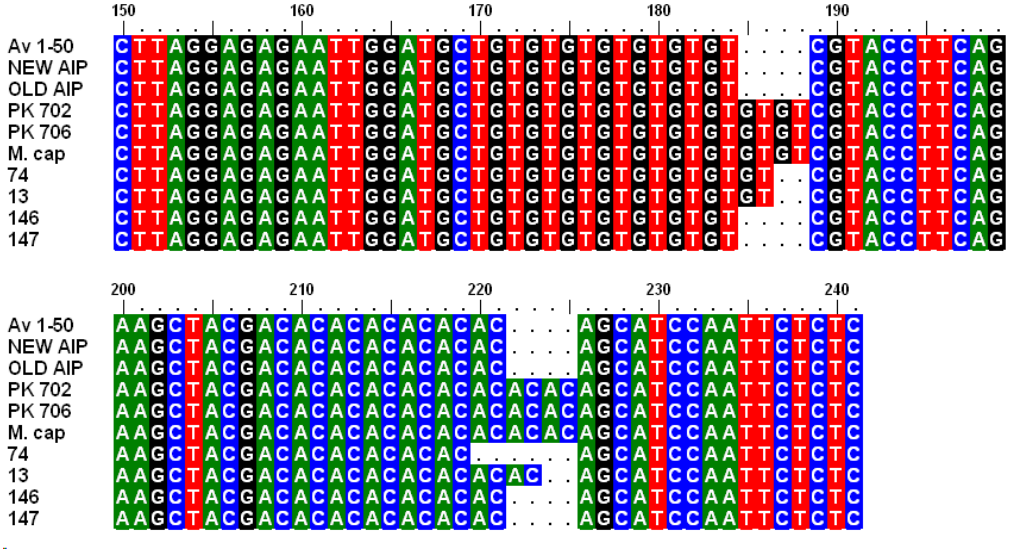


**Figure 18 -** Sequences alignment of the Microsatellite locus B7Sym15 (MS). Av1-50 = consensus sequence of 50 type B1 strains isolated from *Aiptasia pallida* and *Sinularia* flexibilis. NEW AIP - 74 = MS sequences of symbiotic B1 strains, based on MS flanking region. 13 – 147 = MS sequences of potentially free-living B1 strains, based on MS flanking region.

## 3.7. Study of sexual reproduction in clade A, B and C cultures

Following the unsuccessful attempts of documenting sexual reproduction in type B1 cultures from *A. pallida* and *S. flexibilis*, crossing experiments were extended to other B1 cultures and other clades. Specifically, crossing experiments were conducted between cultures of the ITS2-types A1, A2, B1 and C2 (see table 27 for a complete list). The best evidence for the occurrence

of sexual reproduction was found in crossing experiments between cultures 24 and 370 (Fig. 19) and those cultures are both from clade A (Table 17).

**Table 16 –** Genetic characterization of the cultures 24 and 370

| Culture | Query coverage (%) | Identity (%) | GenBank | Clade |
|---|---|---|---|---|
| 24 | 97 | 99 | AF427466.1 | A |
| 370 | 96 | 99 | AF427465.1 | A |

Blast results of complete ITS1-5.8S-ITS2 sequences of cultures against the GenBank database. Shown are search statistics, accession numbers of best matches and the resulting clade/type characterization.

As in previous crossing experiments with type B1 cultures, the formation of smaller zoospores through binary fission of regular sized zoospores could be observed (Fig. 19 A and B). However, in crosses between 24 and 370 these smaller zoospores were attracted to regular sized zoospores and would swarm around them (Fig. 19C). The actual attachment of small and large zoospores could not be observed but pairs of small and regular sized zoospores were documented (Fig. 19D), which gave a first indication of anisogamy in *Symbiodinium* sp. The pairs remained motile for several hours and eventually settled and started what appeared to be a fusion process (Fig. 9E). The process was very slow and after 4 days was still incomplete. Still, these observations constituted the best evidence for sexual reproduction in *Symbiodinium* spp., so far.

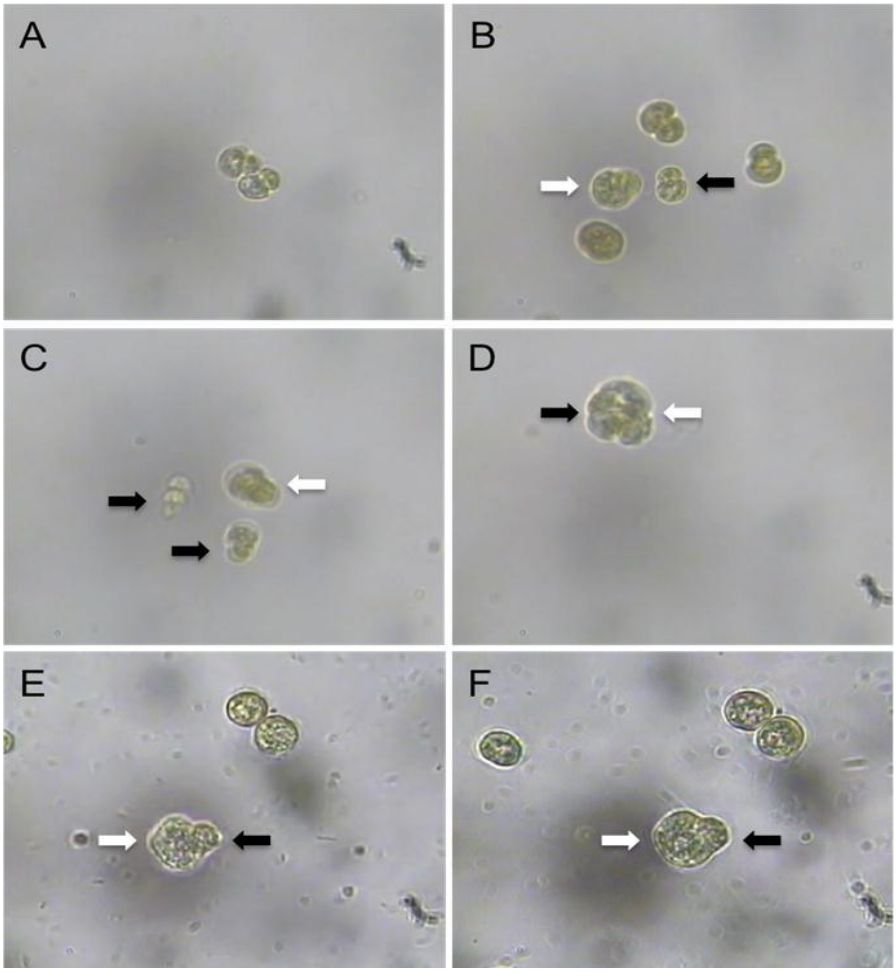


**Figure 19 -** Sexual life cycle processes during crossing experiments between the clade A strains 24 and 370. A) A small type of zoospore is formed by binary fission. B) Size comparison of four regular sized zoospores (white arrow) and one small zoospore (black arrow). C) Two small zoospores (black arrows) swarm around a regular sized zoospore (white arrow). D) Small (black arrow) and large zoospore (white arrow) attached to each other. E) Advanced stage of cell fusion between small (black arrow) and large zoospore (white arrow). F) Late stage of cell fusion between small (black arrow) and large zoospore. Magnification = 400 x.

### 3.8. Cell-cell interactions between *Symbiodinium* spp.

Crossing experiments revealed direct cell-cell interactions between three distinct pairs of cultures. The original ITS-genotyping of the strains in question suggested that the observed interactions were at the inter- and intra-cladal level. However, some of the interacting strains had not been genotyped for long periods of time and so questionable strains were re-typed (Table 17). Sequence analysis of their ITS-region confirmed that the interactions were at the inter- and

intra-cladal level but also resulted in a new classification of culture 154 as a B16 strain and not as an A2 strain as originally classified.

**Table 17** – Genetic characterization of the strains 12, 97, 99, 130 and 154.

| Culture | Query coverage (%) | Identity (%) | GenBank | Clade/Type |
|---|---|---|---|---|
| 12 | 96 | 100 | EU074864.1 | B1 |
| 97 | 97 | 100 | AF427468.1 | A |
| 99 | 97 | 100 | AF427468.1 | A |
| 130 | 97 | 100 | AF427468.1 | A |
| 154 | 98 | 95 | DQ174724.1 | B16 |

Blast results of complete ITS1-5.8S-ITS2 sequences of cultures against the GenBank database. Shown are search statistics, accession numbers of best matches and the resulting clade/type characterization.

Three distinct types of cell-cell interactions could be observed: a) between zoospores of a type-A1 strain (culture 61) and vegetative cells of a type-B1 strain (culture 12), b) between the zoospores of two clade A strains (cultures 99 and 130) and c) between the zoospores of a clade A strain (culture 99) and the zoospores of a type B16 strain (culture 154).

a) Following the crossing of the cultures 61 (type A1) and 12 (type B1), A1 zoospores removed their transverse flagellum from the cingulum (Figure 20A), suggesting that the zoospores detected the presence of the B1 strain. Within minutes after the crossing of the cultures, A1 zoospores attached to B1 vegetative cells with their transverse flagellum (Figure 20B) and paired up with them (Figure 20C). Once attached, the zoospores moved around the vegetative cells in a circular motion, maintaining direct contact with the vegetative cells by their transverse flagellum (Figure 20D). This circular motion could last for up to several hours. Following, the zoospores either separated from the vegetative cells or ceased to move (Figure 20E). The zoospores then rounded up and increased considerably in size, creating extended transparent regions in the cytosol (Figure 20F). In order to prove that this interaction was between the different strains and not between zoospores and vegetative cells of the same strain, further crossing experiments were performed in which the A1 strain had been DAPI life-stained before the mixing of cultures (Figure 21). This selective staining of only one of the interacting strains clearly showed that pairs always

consisted of unstained vegetative cells and stained zoospores (Figure 20 G & H).

b, c) The culture pairs 99 (clade A) with 130 (clade A) and 99 (clade A) with 154 (type B16), formed dense clouds of zoospores within minutes after crossing (Figure 23 & 24). Within these clouds strain 99 appeared to attack the other strains (130 and 154, respectively), which rapidly led to an immobilization of the attacked strain and the sinking of the immobilized cells to the bottom. Once the clouds were deprived of the attacked strain, the aggressor strain dispersed and the clouds disappeared. Left behind stayed a round spot or a trail of immobilized cells, depending on whether the cloud had remained stationary or had moved around.

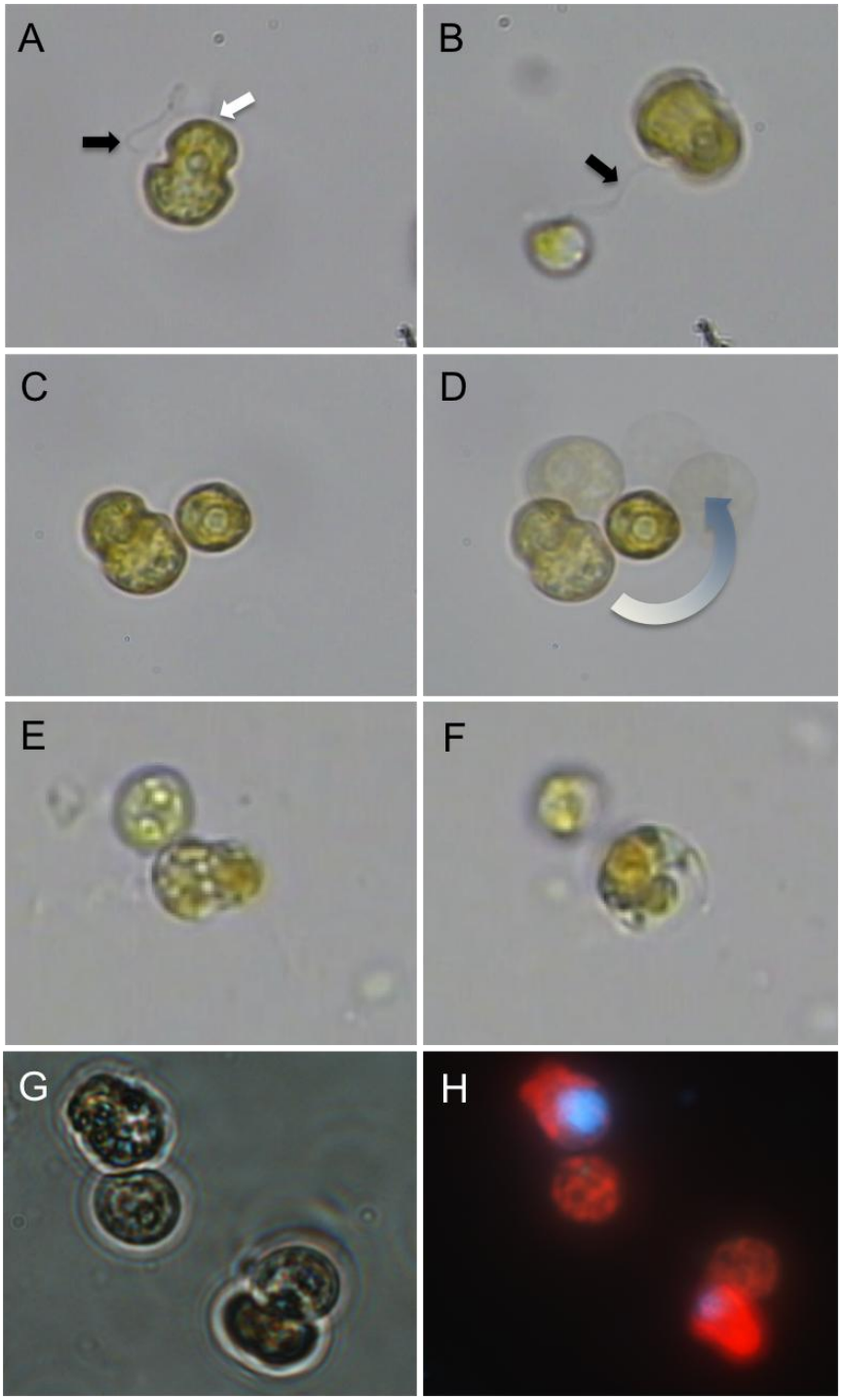


**Figure 20 -** Examination Cell-cell interaction between *Symbiodinium* type A1 and B1 cultures. A) Zoospore of A1 with transverse flagellum removed from the cingulum (black arrow). The white arrow points out the base of the longitudinal flagellum. B) A1 zoospore in contact with B1 vegetative cell through the transverse flagellum (black arrow). C) Paired zoospore and vegetative cell. D) Overlay of four still frames, showing the circular movement of a zoospore around a vegetative cell. E) Zoospore that ceased to move. F) The same zoospore as in E) rounding up and swelling. G) Two pairs of zoospore and vegetative cell as seen under the light microscope. H) The same pairs of cells as in G) under the epifluorescence microscope, showing that the pairs consist of a stained zoospore and an unstained vegetative cell. (Excitation wavelength = 330-380 nm; Emission wavelength = 420 nm)

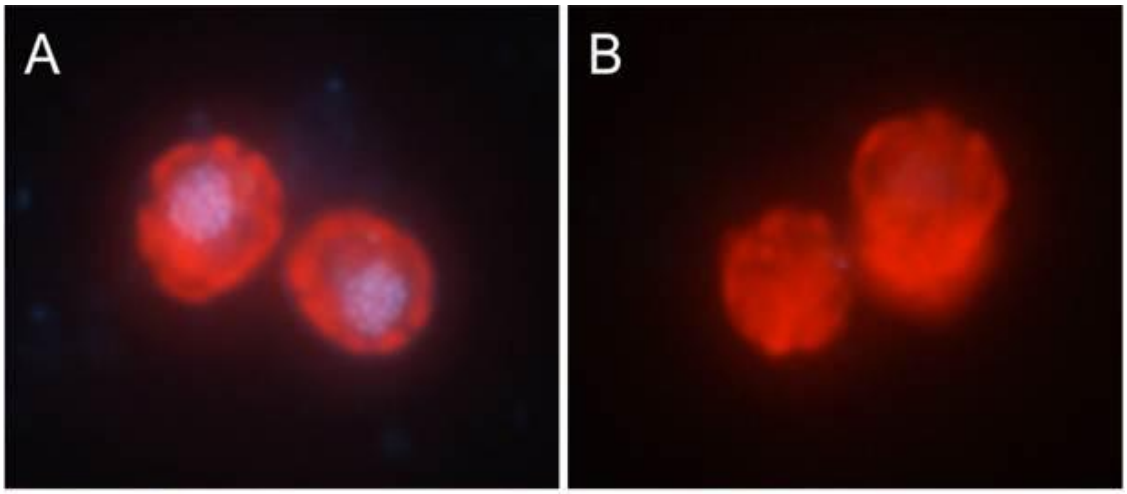


**Figure 21** - Examination of cell-cell interactions between a type A1 and a type B1 strain using DAPI live staining. A) DAPI stained type A1 culture. B) Unstained type B1 culture. Cells as seen under the epifluorescence microscope (Excitation wave length = 330-380 nm; Emission filter = 420 nm).

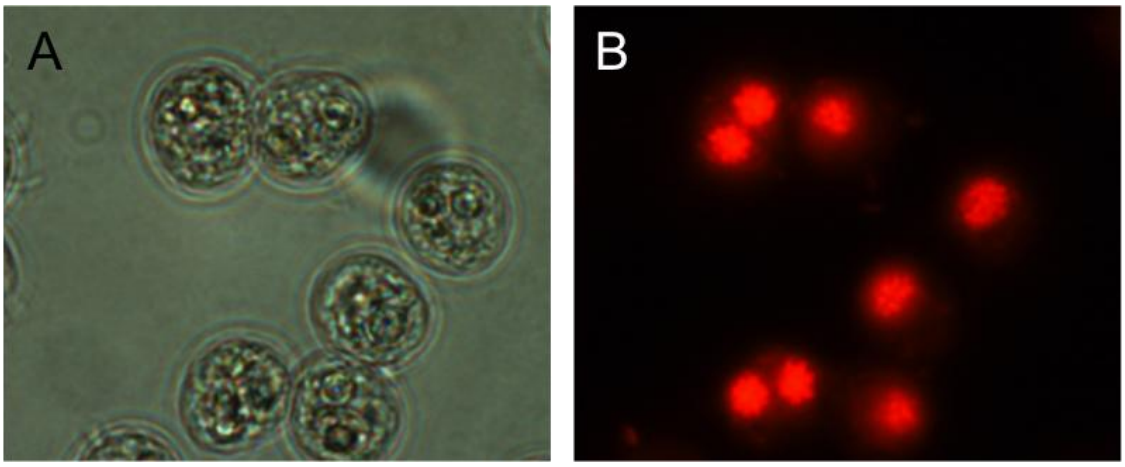


**Figure 22 -** Propidium iodide staining of *Symbiodinium* sp. A) Light microscopic image of stained *Symbiodinium* sp. cells. B) The same cells as in A under the epifluorescence microscope (Excitation wave length = 510-560 nm; Emission filter = 590 nm)

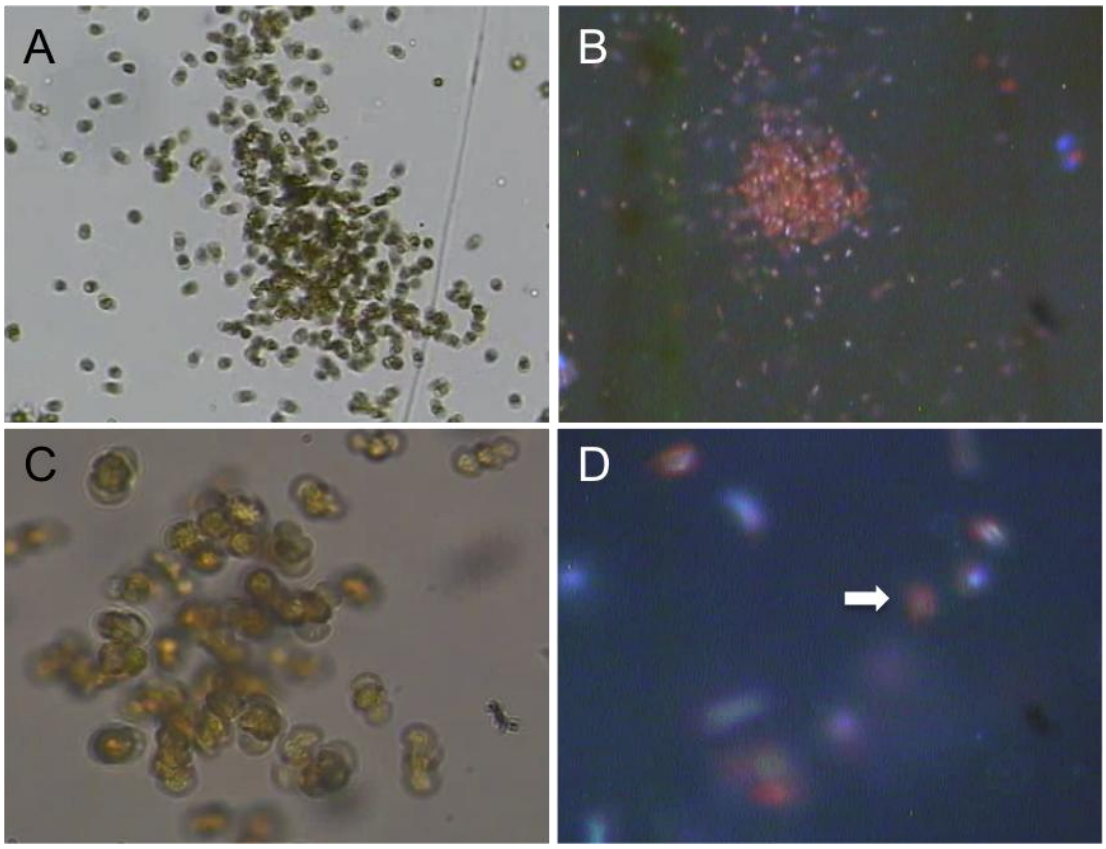


**Figure 23 -** Examination of cell-cell interactions between a type A1 strain (99) and a type B16 strain (130) using DAPI live staining. A) Cloud formation within minutes after mixing of the cultures as seen under the light microscope. B) Epifluorescence image of the cell cloud shown in A). C) Cell cloud at higher magnification as seen under the light microscope. D) Stained cells (strain 99) and unstained cells (strain 130; arrow) under the epifluorescence microscope. All images are still frames of video footage. Epifluorescence excitation wave length = 330-380 nm; Emission filter = 420 nm).

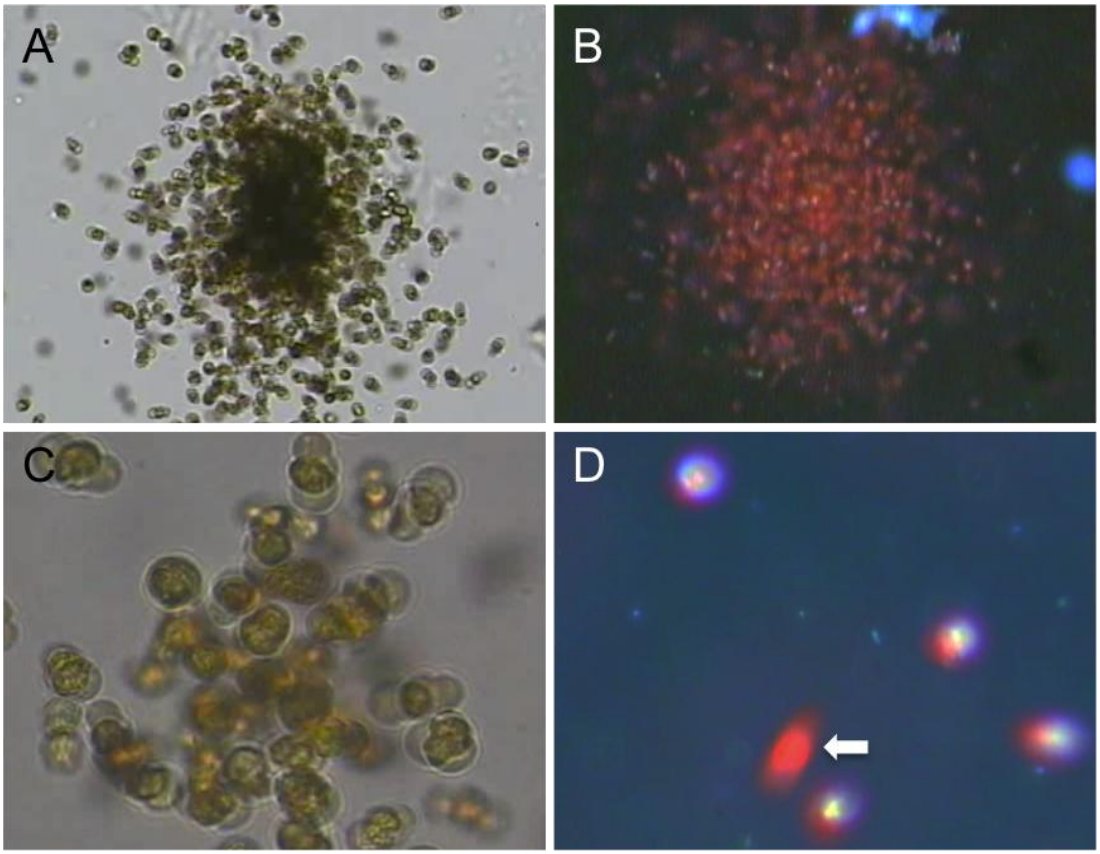


**Figure 24 -** Examination of cell-cell interactions between two distinct type A1 strains (strains 99 and 154) using DAPI live staining. A) Cloud formation within minutes after mixing of the cultures as seen under the light microscope. B) Epifluorescence image of the cell cloud shown in A). C) Cell cloud at higher magnification as seen under the light microscope. D) Stained cells (strain 99) and unstained cells (strain 154; arrow) under the epifluorescence microscope. All images are still frames of video footage. Epifluorescence excitation wave length = 330-380 nm; Emission filter = 420 nm).

### 3.9. Effects of cell-cell interactions on the growth of cultures: Method development

In order to study the effects of cell-cell interactions on the growth of the interacting cultures, a phenotypic difference needed to be identified that would allow the identification of the different strains in co-culture. Propidium iodide staining of nuclear DNA (Figure 1) followed by flow cytometric analysis revealed clear differences in the DNA content between the interacting strains (Figure 2), showing that DNA content could be a suitable phenotypic difference for the identification of strains in co-culture.

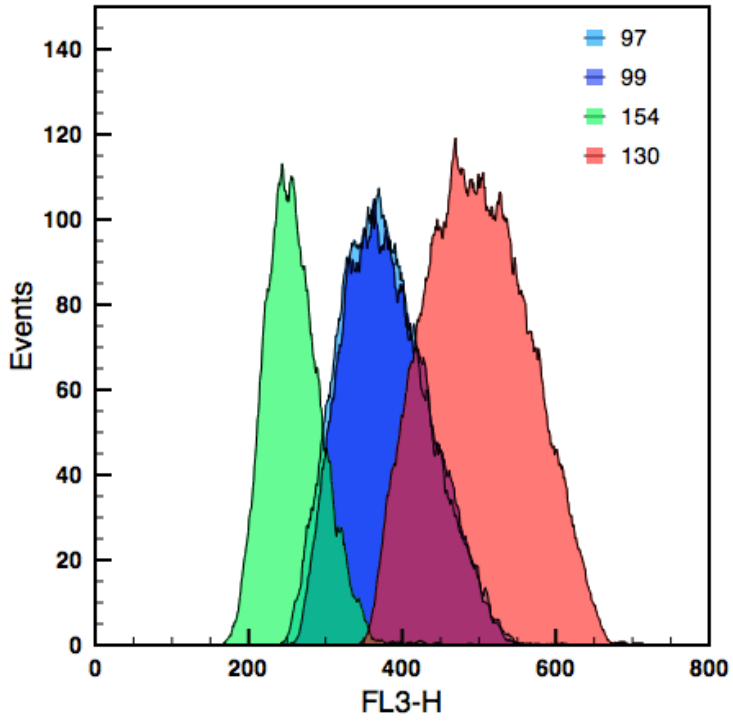


**Figure 25 -** Relative propidium iodide fluorescence (FL3-H) of gated haploid (1n) cells from the *Symbiodinium* strains 97, 99, 130 and 154 as detected by flow cytometry. The propidium iodide fluorescence is proportional to the DNA content and thus reflects relative differences in genome size of the different strains. Each gated 1n population >10.000 events.

Still, in order to quantify total cell numbers of interacting strains, not just 1n populations had to be quantifiable but all other subpopulations with different ploidy levels as well. As shown in figure 26, propidium iodide fluorescence

histograms of different strains in co-culture were more complex than gated 1n populations in isolation. Only through the deconvolution of flow cytometry data, could the different strains be quantified sufficiently well to study the effects of cell-cell interactions on growth characteristics (Figure 26).

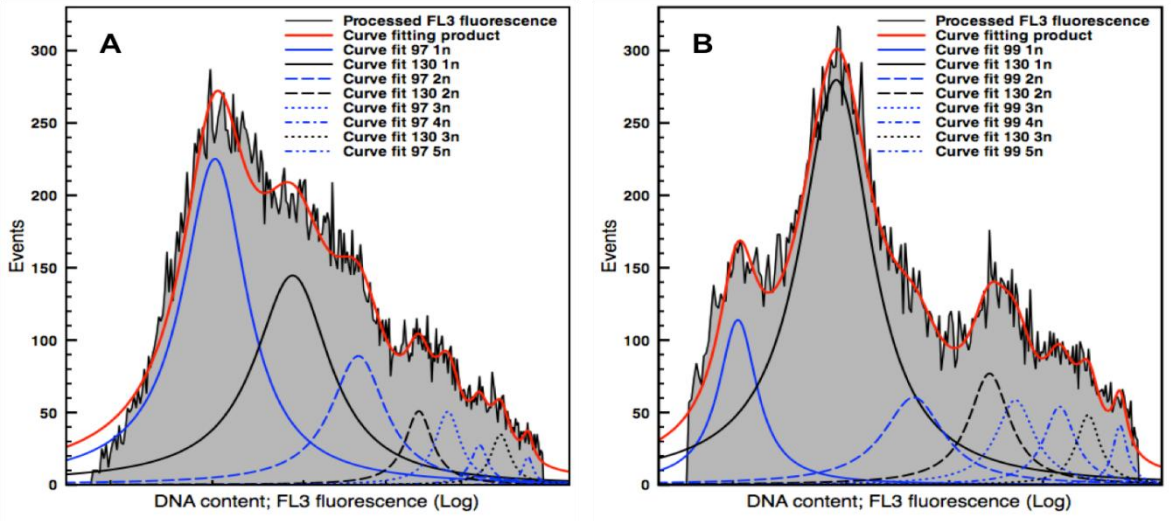


**Figure 26 -** Deconvolution of flow cytometric FL3 fluorescence data by curve fitting of Gauss-Lorentz functions in the software Plot v. 1.997. The fitted curves were integrated to determine the number of events A) Cross between the strains 97 and 130. B) Cross between the strains 99 and 130. The number of fitted curves was based on the fluorescence signal of the 1n peaks and their expected multiples (2n, 3n, 4n, 5n) up to the fluorescence maxima of the processed data. Processing of the raw FL3 fluorescence data to remove debris and cell clusters was performed in the software Flowjo before importing the processed data into Plot.

## 3.10. Effect of cell-cell interactions between the strains 99 and 130 on culture growth

In order to distinguish between the effects of nutrient competition - expected between any two strains of *Symbiodinium* spp. - and the specific effects of the cell-cell interaction, a suitable negative control was needed. This control was found in strain 97. As shown earlier, this strain shares an identical ITS1-5.8S-ITS2 sequence (Table 17) and an identical DNA content (Fig. 25) with strain 99, suggesting that 97 and 99 are closely related strains. However, the cell-cell interactions that were observed between 99 and 130 could not be

observed between 97 and 130, despite several crossing experiments between these strains.

The maximum growth rates of 97 and 130 in isolation were 0.415 and 0.286, respectively. Unexpectedly, the same two strains in co-culture reached a total maximum growth rate that was higher than each of the cultures grown in isolation (r=0.436). In stationary phase, which was reached around day 15-20, strain 97 in isolation reached higher cell concentrations than strain 130 in isolation (Fig. 18A and 19). Compared to this, the maximum total cell numbers of both strains in co-culture reached intermediate values.

Similarly to the previous case, strain 99 reached the higher number of cells when in stationary phase than strain 130 (Fig. 18 B). The crossed culture (99*130) reached an intermediate maximum cell concentration. Strain 130 had a lower growth rate (r=0,286) than strain 99 (r=0,326). Again, the crossed cultures achieved a higher growth rate (r=0,354) than the two cultures in isolation.

Although this phenomenon is not clear, a possible explanation to the higher growth rates in the co-cultures is that low levels of stress can be stimulatory or even beneficial for various growth parameters. This phenomenon is termed as the “Hormesis Effect” and it is gaining increased recognition as evidence accumulates (Cedergreen et al., 2009; Duke et al., 2006).

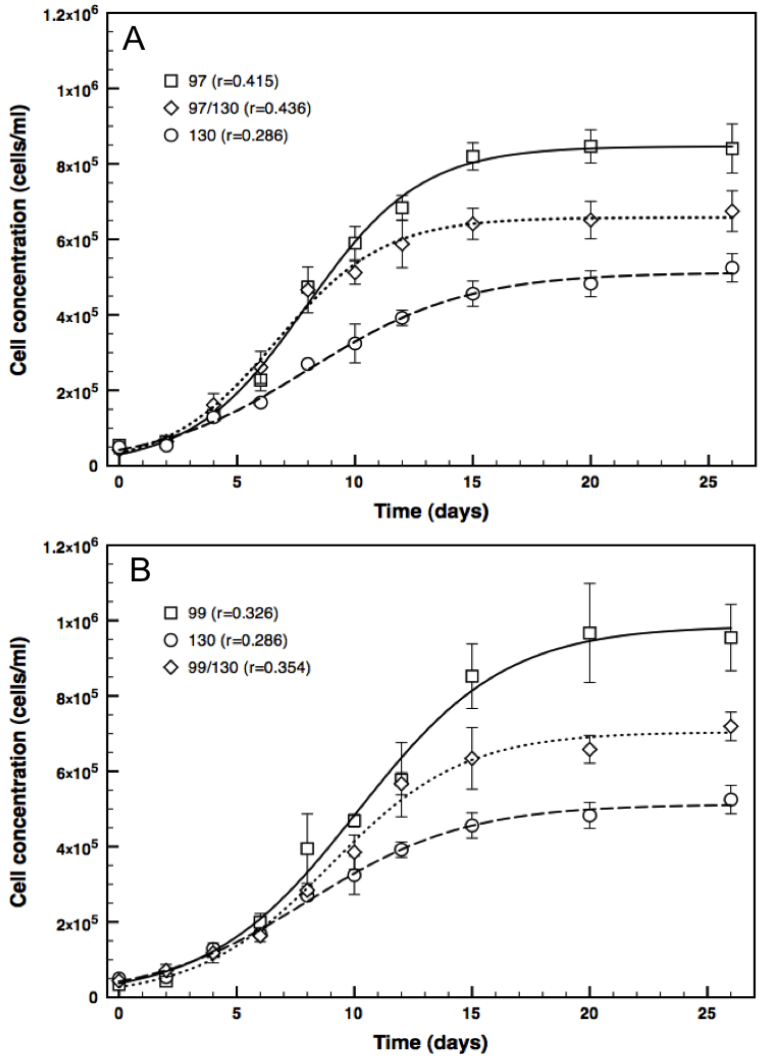


**Figure 27 -** Growth curves of the strains 97, 99 and 130 both in isolation and in co-culture (n=3). A) 97 and 130 isolated and 97/130 in co-culture. B) 99 and 130 isolated and 99/130 in co-culture. Logistic curves were fitted using the software Plot for Mac (v. 1.997). Growth rates were determined by the curve fitting process.

Based on the DNA content of each *Symbiodinium* strain, it became possible to perform the calculation percentage of each strain present in the mixed culture for each time point (Fig. 20).

In the cross between cultures 97 and 130 (Fig. 27 A), culture 97 presents higher maximum population density than culture 130. Growth rate of culture 97 in the presence of culture 130 (r=0,504) is higher than the growth rate of culture 130 in the presence of culture 97 (r=0,191). Culture 97 (in presence of culture 130) has a higher growth rate (r=0,504) if compared to the monoclonal culture

of 97 (r=0,415)(Fig 27 A). Regarding culture 130, it shows a lower growth rate (r=0,191) in the presence of culture 97 when compared to the monoclonal culture of 130 (r=0,286)(Fig 27 A).

In the cross between cultures 99 and 130 (Fig. 27 B), culture 130 reached a higher maximum population density than culture 99. The growth rate of culture 130 in the presence of culture 99 (r=0,381) was lower than the growth rate of culture 99 in the presence of culture 130 (r=0,395). Though, growth rate of culture 130 in the presence of culture 99 (r=0,381) was much higher than the growth rate of the monoclonal culture 130 (r=0,286)(Fig. 27 B). Regarding culture 99, it showed a higher growth rate (r=0,395) in the presence of culture 130 when compared to the monoclonal culture of 99 (r=0,326)(Fig. 27 B).

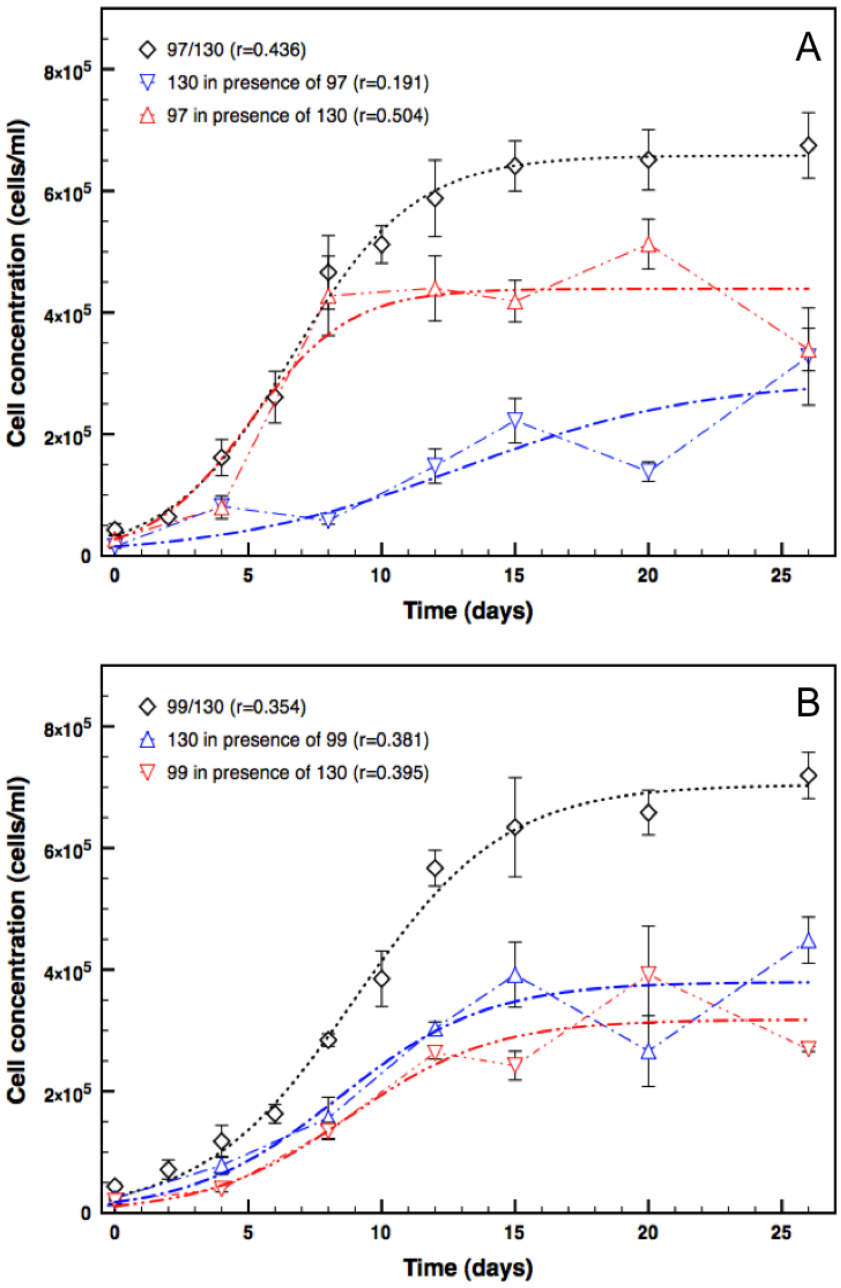


**Figure 28 -** Growth curves of the strains 97/130 and 99/130 in co-culture after deconvolution of flow cytometry DNA content data (n=3). A) Growth curves of 97/130 in co-culture (as in figure 27A) and of the two strains after deconvolution. B) Growth curves of 99/130 in co-culture (as in figure 27B) and of the two strains after deconvolution. Logistic curves were fitted using the software Plot for Mac (v. 1.997). Growth rates were determined by the curve fitting process.

Resorting to extrapolation of the effect that different strains of *Symbiodinium* have on each other when grown in co-culture, the competition coefficient can be calculated. The competition coefficient of strain 97 in the presence of strain 130 (1.482) is bigger than the competition coefficient of strain 130 in the presence of strain 97 (0.535)(Fig. 30 A). The competition coefficient of strain 99 in the presence of strain 130 (1.745) is bigger than the competition coefficient of strain 130 in the presence of strain 99 (0.412)(Fig. 30 B). Curve fitting of the monoclonal growth curves allows for a better visual comparison.

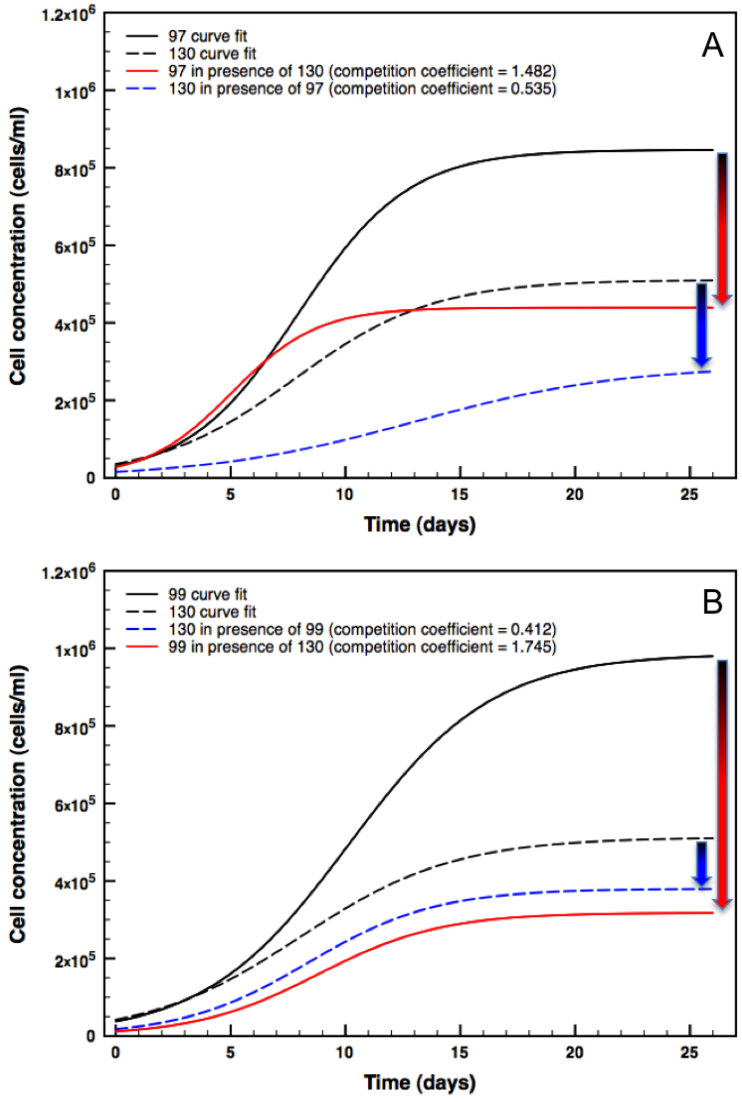


**Figure 29 -** The effect of competition between the strains 97/130 (A) and 99/130 (B) on the maximum population density at equilibrium. Based on the maximum population density in isolation (K) and the population density at equilibrium with a competitor (Neq), the competition coefficient (α) can be determined as: α = (K-Neq)/Neq competitor. An α of 0 means no competition. The larger α gets, the more is the population affected by the competitor.

# 4. Discussion

## 4.1. First genetic characterization of cultures from *A. pallida* and *S. flexibilis*

The aim of the first genetic screening was to determine the identities of the cultures isolated from *Sinularia* flexibilis and *Aiptasia pallida*. Three widely used ribosomal markers were used for this purpose. The fact that the 18S region could not be amplified from any of the screened cultures must have been due to PCR-related problems since both the 28S and the ITS regions could be amplified successfully from the same DNA templates. The 28S region is generally more conserved than the ITS region due to functional constraints in the ribosome. In *Symbiodnium* spp., the 28S region is commonly used as a genetic marker to discriminate the different clades but it is not suitable for discriminating the diversity within clades (A. C. Baker et al., 1997; LaJeunesse, 2001; McNally et al., 1994; Rowan et al., 1991). In this study the 28S allowed cultures to be identified as clade B. Only the less conserved ITS region, which does not encode functional components of the ribosome and is under less selective pressure, allowed the discrimination of the samples down to the type level. The fact that all 8 randomly selected samples were of the same type B1, suggested a dominance of this type in the culture collection. For the four cultures isolated from *Aiptasia pallida* this result was not surprising because this type often dominates this species (Diekmann et al., 2003). More surprising was that the four screened strains isolated from *Sinularia* flexibilis were also of type B1 because this coral species usually harbours clade C strains (Howells et al., 2009; Thornhill et al., 2009). The dominance of type B1 in *S. flexibilis* could have been due to an artificial selection process in culture. However, it was not the focus of this work to study this aspect further. Also, the lack of detailed information about the history of coral colonies in use would have hampered any efforts in addressing this question.

For the study of sexual reproduction, the apparent dominance of B1 strains in the established culture collection meant that efforts had to be restricted to a single clade and type but it also meant that a large number of strains of the same type were available for experimentation. Still, how the dominance of a single ITS-type would affect the chance of success in identifying sexual life

cycle processes was not clear since relations between ITS-types and biological species are not known for *Symbiodinium* spp. By definition, it is the level of the biological species at which sexual reproduction processes occur. In a genus as morphologically indistinct as *Symbiodinium* a genetic marker that discriminates well at the species level would be extremely useful in that it could help in targeting promising strains for the study of sexual life cycle processes. Unfortunately, genetic tools to discriminate *Symbiodinium* spp. effectively at the species level are not available. This is not so much due to a lack of genetic markers that are discriminative enough, since microsatellite markers and SNPs are well established in the study of *Symbiodinium* diversity (Carlon et al., 2008; Frommlet et al., 2008; Pettay et al., 2007). More challenging is that the lack of distinct phenotypic features and of a biological species concept imped efforts in uniting information on genetic diversity with a species concept in this diverse genus.

### 4.2. Known vegetative life cycle stages of *Symbiodinium* spp.

In this study, all the vegetative life cycle stages of *Symbiodinium* spp. described in the literature were found, apart from the aplanospore and the binary fission of the vegetative cell (Freudenthal, 1962; Stat et al., 2006). The aplanospore is only found in cultures grown on solid medium and the binary fission of vegetative cells is described only *in symbio* (Freudenthal, 1962). Thus, both cases were out of the scope of the present study. The fact that the study of the life cycle of *Symbiodinium* spp. started by the identification of the known vegetative life cycle stages built the basis needed for the identification of novel life cycle stages, either vegetative or sexual. This stage of the work focused only in the microscopic observations of the cultures and the documentation of the several life stages.

### 4.3. Novel life cycle stages

One major change to the described life cycle of *Symbiodinium* spp. is that the division process mostly gives origin to one or two motile zoospores, instead of giving origin to non-motile vegetative cells. *Symbiodinium*'s life cycle has been described for the first time about 50 years ago and the hypothesis of sexual reproduction within its life cycle hasn't been confirmed ever since.The supposed sexual life cycle stages proposed by Freudenthal (1962) were also found. Indeed, molecular analyses on several biomarkers dictate that *Symbiodinium* spp. is likely to have a sexual stage in its life cycle (Baillie et al., 2000; Goulet et al., 1997; Schoenberg et al., 1980a).

In addition to the vegetative life cycle stages described in the literature (Freudenthal, 1962), four new life cycle stages of *Symbiodinium* spp. were identified and documented. In one isolated case, an exceptionally large motile zoospore was documented emerging from its zoosporangium. Although it was documented in a clonal strain (culture 24A3, type B1), it is uncertain that the respective culture remained uncontaminated and this large zoospore could be the result of gamete fusion, as it happens in other dinoflagellate species (Blackburn et al., 1989). Contrary to Freudenthal (1962), who stated that free-living zoospores do not divide along the girdle or along any other exterior demarcation, several zoospores were observed dividing longitudinally by binary fission (Fig. 19A). Whether the resulting, smaller zoospores were vegetative cells or another life cycle stage could not be clarified at this stage. However, later observations in other cultures indicated that zoospores resulting from binary fission are gametes as they displayed attraction towards regular sized zoospores and fusing pairs of small and large zoospores could be documented (more detail on the section "Study of sexual reproduction in clade A, B and C cultures"). The fusion process between different sized cells suggests that *Symbiodinium* spp. produces anisogametes. The fusion process of the described anisogametes was followed over four days but did not reach complete fusion of the cells into a planozygote. Thus, none of the life cycle

processes that typically follow dinoflagellate planozygote formation, such as dormancy period and fate of the emerging planomeiocyte following dormancy, could be studied thus far.

Based on the findings of this study, a modified and extended *Symbiodinium* spp. life cycle is proposed (figure 30). The basis for this extended life cycle is the one proposed by Freudenthal (1962) and its original information was preserved when indicated. The normal vegetative cell gives origin to one or two zoospores. The doublet zoosporangium may, in turn, divide again to give origin to the rare tetrad cells which, upon rupture, release four motile zoospores. In clonal cultures of *Symbiodinium* type B1, direct division of zoospores was repeatedly observed, though their origin is still not clear. The fact that they were observed in a clonal culture suggests that this stage is part of the vegetative life cycle of *Symbiodinium* sp. However, this direct division of the zoospores was also observed in the crossing experiments between cultures 24 and 370 which culminated in the fusion of these smaller freshly divided zoospores with regular sized zoospores, suggesting that the direct division of the zoospores is the phenomenon through which the smaller gametes are produced. However, the origin of the regular sized gametes is uncertain. During the fusion process, the pair of gametes remained motile for several hours, until they finally settled. This stage is suggested to be diploid and its upcoming cellular events are not known. It may give origin to a bigger zoospore (as the one observed in the culture 24R3) which, in turn, must divide in order to give origin to two new haploid cells. Future work is still needed to elucidate clearly the unknown origins and fates of the newly discovered stages of the life cycle of *Symbiodinum* spp.

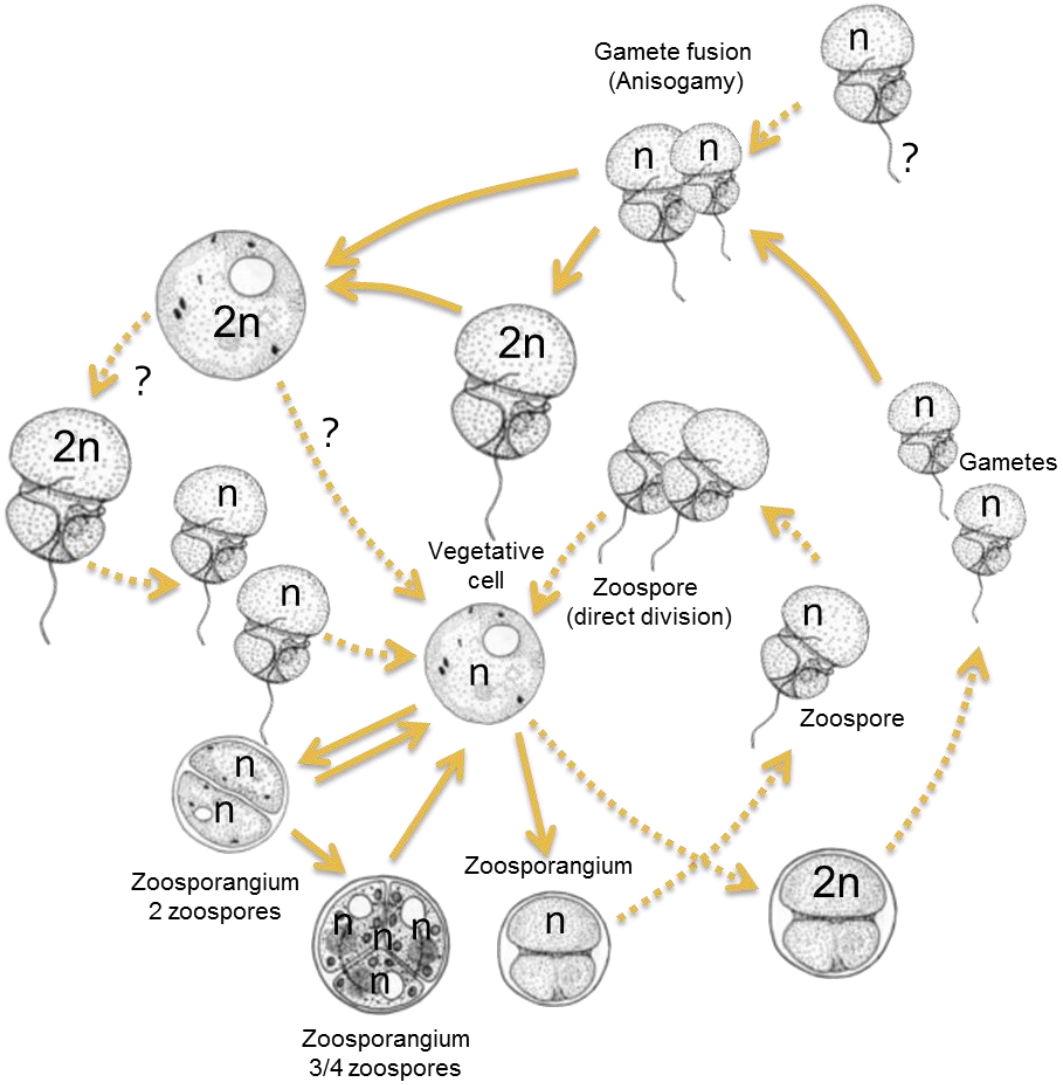


**Figure 30** - Proposed life cycle of *Symbiodinium* spp. Continuous arrows described the known stages for *Symbiodinium spp.* life cycle. Dashed arrows indicate the probable origins of the gametes for this species. Although the certain origin of the gametes remains unknown, strong attractions found between different size cells led to the improvement of the life cycle, including some omitted stages so far.

### 4.4. Study of sexual reproduction in type B1 cultures

As mentioned earlier, it is the level of the biological species at which sexual reproduction occurs but suitable phenotypic features and genetic markers to discriminate *Symbiodinium* spp. at this level are still missing. Due to the ill-defined species-concept in genus *Symbiodinium*, crossing experiments, which are so commonly used to study sexual life cycle processes in other dinoflagellate species (Blackburn et al., 1989; Destombe et al., 1990) are particularly challenging with these dinoflagellates. The fact that crossing experiments within the type B1 cultures did not result in any clear signs for sexual reproduction between the putative gametes, zoospores and vegetative cells could thus have been due to a whole range of reasons. Regarding the putative gametes all efforts of clarifying their identity failed and it is unlikely that the inability to attain any indications for sexual reproduction between these cell-like bodies was due to reproductive strain incompatibilities as described in the following section. The failure of amplifying rDNA sequences with *Symbiodinium*-specific primers together with the failed attempt to stain the DNA with propidium iodide makes it very unlikely that these bodies were any *Symbiodinium* spp. cell type. One possibility is that these bodies were a fungal contaminant. Blank (1987) already suggested that Freudenthal (1962) may have misidentified fungal spores as potential gametes and Professor Jorge Rino from the Department of Biology, University of Aveiro, also suggested these bodies to be fungal spores of *Chytridia* sp., a unicellular fungus that infects microalgae (personal communication). If this was the case, it remains intriguing that propidium iodide did not result in any visible nuclear staining. The absence of gametes in the crossing experiments could also be an explanation for the unsuccessful tries. The conditions that trigger gametogenesis in other dinoflagellate species, such as ROS (A. M. Nedelcu et al., 2003a) are still not unravelled for *Symbiodinium* spp. and thus, could not be mimicked. The analysis of the microsatellite locus B7Sym15 and its flanking region, culminated in the likelihood of clonal origin for all the cultures utilized in the initial crossing experiments. Therefore, the existence of mating-types was compromised and there was no interaction observed.

In order to genetically characterize in more detail the isolated cultures of the culture collection used for crossing experiments, the analysis of the microsatellite locus B7Sym15 and its flanking region was performed, resulting in the likely clonal origin of the cultures. Therefore, the cultures used for the study of sexual reproduction in *Symbiodinium* type B1 were incompatible, what justifies the lack of sexual reproduction on the crossing experiments. In fact, the low diversity of clade B populations of *Symbiodinium* spp. in corals that divide by asexual reproduction (i.e., fragmentation) is thought to be a bias of sampling the same genetic individual multiple times in such corals (Thornhill et al., 2009). The main ways of transmission of symbionts is dependent on the species (vertical transmission vs. horizontal transmission). In laboratory, as a consequence of the frequent fragmentation of the corals cultured, a certain type of symbiont might be favoured as a bias of vertical transmission. As a result, the corals that harboured the cells sampled within the scope of the present work which have been kept in the aquarium for a long time and are often fragmented, probably led to an artificial selection process of the symbionts.

### 4.5. Study of sexual reproduction in clade A, B and C cultures

Several species of dinoflagellates have distinct types of gametes: a) hologamous gametes, when the reproductive cells do not differ from vegetative cells, b) isogamous gametes, when the reproductive cells are morphologically different from the vegetative cells (by size and/or amount of pigments) but are identical among themselves and c) anisogamous gametes, when the gametes are both different from the vegetative cells and among themselves (Pfiester, 1987). Since there was no previous record of sexual reproduction in *Symbiodinium* spp., there was also no record of the type of gametes that might be produced (Dubinsky & Stambler, 2011). Thus, in the search for sexual reproduction in the cultures provided by Mark Warner's lab (including clades A1, A2, B1 and C2) attention focused not only on one cell type, but on all that could be indicative of the presence of gametogenesis.

In one of the crossing experiments (cultures 24 and 370), cell division by means of binary fission of the zoospores could be observed. In addition to observations of the same type of cell division in type B1 cultures, in this cross the resulting smaller zoospores could be observed to be attracted to regular sized zoospores. Several pairs of small and regular sized zoospores could be observed. The pairs would remain motile for several hours and then settle down and continue to what appeared to be a fusion process. The resting-cyst is a common phenomenom of sexual reproduction among dinoflagellates, and the amount of time that it rests in this stage varies according to the species (Figueroa, Garces, & Bravo, 2007; Kokinos et al., 1998; Pfiester, 1987). The putative planozygote observed in this cross did not completed the fusion process over a period of four complete days, and post-zygotic division could not be observed due to the fact that the fusion process was likely to be still occurring. Thus, further research with these cultures will give clearer elucidation about the process of sexual reproduction within *Symbiodinium* spp. Still, the results obtained during the course of this work, suggested this to be the first visual indication for the existence of a sexual stage in the life cycle of *Symbiodinium* spp.

According to the literature, sexual reproduction seems likely to occur in clade A (type A1) symbionts (Baillie et al., 2000). Belda-Baillie et al. (1999) inoculated aposymbiotic larvae of *Tridacna gigas*, with *Symbiodinium* sp. clonal strains and found a higher genetic variability in the symbiotic populations of the 3-month old clams then they had been infected with. Such results indicate that genetic recombination processes had occurred among the symbiotic populations as a result of sexual reproduction. Again, indirect evidence of sexual reproduction in *Symbiodinium* spp. accumulates and points towards the existence of a sexual life cycle stage in this dinoflagellate.

### 4.6. Cell-cell interactions between *Symbiodinium* spp.

In nature, no population exists in isolation and co-habiting populations interact in multiple ways and often complex ways. If two species co-habit, they may interact in six ways (such interactions are described by the combinations of the following symbols “0”, “+” and “-“): 0 means that the species is not affected by the presence of the other, + means that the species is favoured by the presence of the other and – means that the species is impaired by the presence of the other. Neutral interactions are described by 0/0, negative interactions are described as -/-, 0/- or +/- and lastly the positive interactions are described as +/0 or +/+. Keddy (1989) described competition between organisms as a negative effect of one over the other, either by consuming or controlling the access to a limiting resource. Aside from this indirect- or exploitation competition, direct- or interference competition involves direct interactions between individuals (e.g. aggression) that interfere with e.g. survival, reproduction or physical establishment in the habitat. Direct competition is evolutionarily detrimental if there is no subjacent competition for a resource. If one regards the ecology of cnidarians-symbionts ecology, it is legit to regard the host as a resource, once it provides the necessary inorganic nutrients that the symbionts need to photosynthesize (Yellowlees et al., 2008). After a bleaching event where the host is deprived of its symbionts, the recovery from the bleached state is possible by the uptake of free-living *Symbiodinium* spp. from the environmental pool (Brown, 1997; Ove Hoegh-Guldberg, 1999). Free-living *Symbiodinium* spp. should recognize the host and this phenomenon is possible via lectin/glycan recognition molecules (Fransolet et al., 2012) and it should be attracted to them (W. Fitt, 1984; W.K. Fitt, 1985). Thus, it is likely that the motile cells of *Symbiodinium* spp. compete between themselves for the limiting resource that is the host. Such competition would likely culminate in a higher success rate of the re-colonization of the host for the winning strain.

*Symbiodinium* spp. was found to display interactions between some strains. Such interactions are both at the inter- and intra-cladal level. Three kinds of interactions between the strains were observed:

a) Between the vegetative cells of culture 12 (type B1) and the zoospores of culture 61 (type A1);
b) Between the zoospores of culture 99 (clade A) and the zoospores of culture 130;
c) Between the zoospores of culture 99 and the zoospores of culture 154 (B16).

It is clear that some *Symbiodinium* spp. strains interact with each other. Such interactions consisted of cell-cell contact of one strain over the other until the victim strain had lost its flagella and settled on the bottom. The cell contacts observed for both interactions are not sufficient to kill the victim strain, in opposition to the described interactions of other dinoflagellate species (Uchida et al., 1999; Uchida et al., 1995). The mechanisms by which the cells of the victim strain are immobilized are not known. One possible explanation is that the attacker strain produces some inhibitory substance that is attached on the victim upon cell-cell contact. Another possible explanation is that the cells of the victim strain are simply damaged mechanically by contact with the attacker strain. The interactions described in the present work may have ecological important implications for the colonization of hosts by competing *Symbiodinium* spp. strains.

### 4.7. Effect of cell-cell interactions on the growth of cultures: Method development

To study the effect of cell-cell interactions on the growth of the interacting *Symbiodinium* strains, it was necessary to establish a method that would enable the distinction of the different strains and their separate quantification when in co-culture. The DNA content of *Symbiodinium* differs within and between the different clades (LaJeunesse et al., 2005) and thus was a potential target for the development of such a method. Flow cytometric analysis of the interacting strains showed clear differences in DNA content and thus confirmed that DNA content could be exploited as a phenotypic marker to study strain interactions. It is generally accepted that *Symbiodinium* spp. is haploid (Santos et al., 2003),

though, ploidy levels differ with the vegetative cell cycle stage (e.g. tetrad cells display a DNA content of 4n). Consequently, raw flow cytometry data on DNA content of strains in co-culture were complex since peaks of different subpopulations and of both strains overlapped considerably. Therefore, in order to quantify the different strains the data needed to be deconvoluted into the signals of the different underlying subpopulations of cells. By fitting Gauss-Lorentz curves, it was possible to deconvolute the data and subsequent integration of the different fitted curves allowed the interacting strains to be quantified.

### 4.8. Effect of cell-cell interactions between the strains 99 and 130 on culture growth

In order to explore the effects of the interaction seen between the strains 99 and 130 over each other when grown in co-culture, samples of the crossing experiment were analyzed by flow cytometry twice a week, during four weeks. In all the time-point analyses, flow cytometry allowed gathering information about DNA fluorescence and thus, it was possible to study the percentages of each strain during the course of the experiment based on their relative DNA content (Fig. 25). Deconvolution of the data originated by flow cytometric analyses of the co-cultures, revealed itself as the best approach to investigate the growth rates, maximum cell concentrations and competition coefficient of the strains in co-culture. As a comparison for the co-cultures, strains 97, 99 and 130 were grown in isolation. The analyzes of the DNA content of each strain grown in isolation allowed to set the 1n peaks on the software Plot (v1.997). Therefore, for the crossing experiments, the software was able to fit the curves to each strain, according to their DNA content and ploidy of the cells.

### 4.9. Control cross (97 and 130):

This cross was selected as a negative control for several reasons. Culture 97 shares an identical ITS1-5.8S-ITS2 sequence to that of culture 99, meaning that these are closely related strains. However, culture 97 displays no

interaction with the remaining cultures in study (cultures 130 and 154), despite the several crossings performed, in opposition to culture 99 that quickly displays the interaction. Also, the DNA content of culture 97 is identical to the DNA content of culture 99 (Fig 25), therefore allowing proper analyses of the flow cytometry data.

Surprisingly, this crossed culture displayed the higher growth rate when compared to the uncrossed cultures, meaning that the crossed culture reaches the stationary phase earlier than the uncrossed cultures. In isolation, culture 97 reaches the higher maximum cell densities, whereas culture 130 reaches the lowest. The deconvolution of the flow cytometric data allowed building the growth curves for the cultures growing in the cross. Therefore, culture 97 reaches the stationary phase earlier than culture 130 (+- day 12 comparing to +- day 25, respectively). Regarding the effect that each culture has on the other culture, competition coefficient of culture 130 is smaller than the competition coefficient of culture 97, which means that culture 97 is more affected by the presence of culture 130 than otherwise. Either way, it is expected that the cultures have an effect on each other when grown in co-culture, once they will compete for nutrients, which is a limiting factor.

### 4.10. Interacting cross (99 and 130):

Direct interactions between cultures 99 and 130 were observed every time the crossing was made, within a few minutes of the mixing. The patterns of the growth curves of the cultures growing in isolation when compared to the crossed culture are similar to the ones of the control cross. Culture 99 (similarly to culture 97) reaches the higher maximum cell numbers at equilibrium, whereas culture 130 displays the lower densities and the crossed culture displays an intermediate maximum cell number. After the deconvolution of the data of this cross, the pattern of the growth curves is quite different when compared to the control cross. The growth rate of cultures 99 and 130 is very similar, which means that both cultures reach stationary phase at the same time

(around day 17). When grown in isolation, culture 130 reaches a lower maximum cell number in the stationary phase compared to culture 99 in isolation. Though, when grown in the co-culture, culture 130 reaches higher cell numbers at equilibrium compared to culture 99. Growth rates of culture 99 in isolation and in the crossed culture are comparable (r=0.326 and r=0.395, respectively) and this suggests that the energy that culture 99 invests in the interaction do not have an implication on the growth of this culture. Tough, the fact that culture 130 reaches higher cell numbers than culture 99 when in co-culture suggests that culture 130 is able to uptake nutrients from the medium more efficiently than culture 99. Another possible explanation for the higher cell numbers of culture 130 in the stationary phase compared to the maximum cell numbers of culture 99 is the fact that some dinoflagellate species are able to compete by allelopathy, by the production of secondary metabolites that affect competing organisms (Vardi et al., 2002)(International Allelophatic Society 1996; available at www.ias.uca.es/bylaws.htm#CONSTI).

# 5. Conclusions and future work

In the present work it was hypothesized that *Symbiodinium* spp. has a sexual stage in its life cycle. The results obtained during the course of the work were consistent with the hypothesis and, therefore it is very likely that *Symbiodinium* spp. is able to undergo sexual reproduction. This aspect of the life cycle of *Symbiodinium* spp. has been proposed fifty years ago and it was not proven thus far, though molecular evidence states the likelihood of this phenomenon (Baillie et al., 2000; Belda-Baillie et al., 1999; Goulet et al., 1997). These findings help to interpret the relevant implications on the cnidarian-symbiont ecology, likely to result in better adapted strains and therefore, more stable relationships between the host and the symbiont (LaJeunesse, 2001). However, more evidence is needed to support this premise.

The second major goal of the present work hypothesized that *Symbiodinium* spp. strains show a competitive behaviour between themselves. Crossing experiments between the interacting strains and the analyses of the interactions by means of flow cytometry resulted in evidence for the competition characterization. It is suggested that culture 99 (clade A) displays an aggressive behaviour when in its free-living stage, towards cell of both the same clade and of other clades. The ecological role of this behaviour is not disclosed, though, it is very likely that it could give an advantage in the recolonization of the host for the aggressor strain 99. Further research on this aspect is required in order to clarify the consequences of this behaviour for interacting strains and effects on host colonization.

In the near future, there are some gaps in the present work that should be cleared. The origin and the fate of gametes and the planozygote should be determined. If there is a hypnocyst, the dormant period should be determined. Strains 24 and 370 should be characterized in further detail as they constitute the first indication for the sexual life cycle in *Symbiodinium* spp. More crossing experiments should be done in order to determine whether sexual reproduction occurs only in clade A strains or if it is common in the whole genus *Symbiodinium*. The identification of the mechanisms that induce sexual reproduction is a goal of great importance, in order to comprehend how gametogenesis is triggered in the environment. Also, an important task is to study sex-related genes, since such genes may allow the development of

alternative molecular markers for the identification of vegetative cells and gametes in natural *Symbiodinium* populations.

As for the direct cell-cell interactions, experiments with the strains 99 and 154 and the control between the strains 97 and 154 have already been performed but data analysis could not be completed in the course of this thesis. Interesting would be to extend future crossing experiments with the aggressor strain 99 to other cultures. The effects of the interaction between culture 12 and culture 61 were not studied yet but it would be important to study this interaction in further detail in order to find out what happens to the zoospores of culture 61 which, after their attachment to the vegetative cells of culture 12, settle down, round up and increase considerably in size. Also, regarding the interactions on *Symbiodinium* spp., it would be interesting to investigate if there is any kind of allelopathic competition between the strains in addition to the cell-cell contact.

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# 7. Annexes

## 7.1. Flow cytometry

**Table 18 –** protocol for flow cytometric analyses

| Reagents |
| --- |
| • Template (culture, the amount depends on the density of the cells)<br>• 4% paraformaldehyde<br>• 1% phosphate saline buffer (PBS)<br>• Methanol<br>• Propidium iodide (3µg.mL-1 in PBS)<br>• RNaseA (1,1 µg.mL-1 in PBS) |
| Protocol |
| 1. Fix the culture with 1% paraformaldehyde for 10 minutes.<br>2. Wash the culture with 1% PBS and remove it by centrifugation at 1200g for 10 minutes.<br>3. Remove the supernatant and add 5mL of cold (4ºC) Methanol (chlorophyll extraction), resuspending the culture and store it for 12 hours at 4ºC.<br>4. Wash the cells twice as described in 2).<br>5. Add 900 µL of PBS and 100 µL of staining solution (50 µL of Propidium Iodide plus 50 µL of RNaseA).<br>6. Store in the dark for 2 hours before analysis. |

## 7.2. PCR protocol

**Table 19 –** Protocol for the DNA extraction, preparation of the mastermix, PCR and electrophoresis.

| **DNA extraction** |
|---|
| 1. Sample 100 µl of the culture for an 1,5 ml eppendorf tube<br>2. Centrifuge for 30-60s<br>3. Remove the supernatant and add 10 µl of ultrapure water<br>4. Add 10-15 sterile glass beads and vortex for 1 minute |

| **Mastermix** | |
|---|---|
| | 1X |
| Taq Polymerase | 0,4 µL |
| dNTP's | 1,0 µL |
| Primers (F/R) | 2,5 µL (of each primer) |
| 10X buffer | 5,0 µL |
| Ultrapure water | 37,6 µL |
| Template | 1,0 µL |
| Total | 50 µL |

| **PCR/Gel electrophoresis** |
|---|
| 1. Run the desired PCR profile<br>2. Assemble the solidification tray<br>3. Prepare 120 mL of 1X TAE<br>4. Weight 2,4g of agarose (to a final concentration of 2%, which was the same as we used before)<br>5. Mix the reagents in an 1L schott bottle<br>6. Boil it in the microwave and, as it starts boiling, turn of the microwave, take out the Schott bottle, mix a bit, and repeat the process, until the solution looks clear<br>7. When the solution is clear, pour it into the used Schott Bottle with Etidium Bromide (2-3 µL). Be careful and do this under the fume hood!<br>8. Pour the liquid into the solidification tray and leave it for at least 45 minutes<br>9. Remove 10 µL of the amplicon and mix it with 2-3 µL of 6X loading dye (each amplicon from each sample)<br>10. Load the sample to the well (the left well is loaded with 5-7 µL of DNA ladder)<br>11. Start the running at 100 V |

## 7.3. F/2 medium

**Table 20** – Recipe of F/2 medium without silicates

| F/2 medium | | | |
|---|---|---|---|
| **Stocks** | | | |
| | **(1)** | **Trace elements (chelated)** | **Per liter** |
| | | Na2EDTA | 4.6 g |
| | | FeCl3.6H2O | 3.15 g |
| | | CuSO4.5H2O | 0.01 g |
| | | ZnSO4.7H2O | 0.022 g |
| | | CoCl2.6H2O . | 0.01 g |
| | | MnCl2.4H2O | 0.18 g |
| | | Na2MoO4.2H2O | 0.006 g |
| | **(2)** | **Viamin mix** | |
| | | Cyanocobalamin (Vitamin B12) | 0.0005 g |
| | | Thiamine HCL (Vitamin B1) | 0.1 g |
| | | Biotin | 0.0005 g |
| **Medium** | | | **Per liter** |
| | | NaNo3 | 0.075 g |
| | | NaH2PO4.2H2O | 0.00565 g |
| | | Trace elements stock solution (1) | 1.0 mL |
| | | Vitamin mix stock solution (2) | 1.0 mL |

**Notes:**

1 – Silicate was not added to the medium, to inhibit diatom growth.

2 – Vitamins are light sensitive and were kept at -20ºC.protected from light with aluminium foil.

3 – Stock solutions were prepared with autoclaved ultrapure water.

4 – To prepare the medium natural sea water was GF/F filtered and autoclaved.

5 – Stored in the fridge (4ºC) and straight before use it should be sterile filtered (0,22 µm).

## 7.4. Lugol's iodine (1 liter)

**Table 21 –** protocol for Lugol's iodine

| Reagents |
| --- |
| • 50g Iodine (granular)<br>• 100g Potassium iodide (granular)<br>• 1000 mL ultrapure water |
| **Protocol** |
| 1. Smash both iodine and potassium iodide with the help of a mortar and pestle.<br>2. Transfer the pouder to an appropriate flask (must avoid metal and plastic containers).<br>3. Fill with ultrapure water and shake the flask thoroughly.<br>4. Store in a dark ambar flask at room temperature. |

## 7.5. 4% paraformaldehyde

**Table 22** – protocol for 4% paraformaldehyde

| Reagents |
| --- |
| • 0,4g of 95% paraformaldehyde<br>• 10 mL of PBS<br>• NaOH 5M |
| **Protocol** |
| 1. Mix 4g of paraformaldehyde (95% ) in 80 mL of ultrapure water<br>2. Warm solution to 56ºC and keep stirring.<br>3. Adjust pH to aprox. 11 with NaOH (+- 50µL 1M per 100mL) |

4. Once the solution is clear, let it gently cool down.
5. Readjust pH to 7,4 with HCL (1M) at room temperature
6. Adjust the volume to 90 mL with ultrapure water
7. Add 10 mL of 10x PBS to a final concentration of 4% paraformaldehyde in 1% PBS.
8. Filter sterilize trough a 0,22 µm filter and store at 4ºC.

## 7.6. 10x phosphate buffered saline

**Table 23** – Protocol for 10X phosphate buffered saline

Reagents

- 80g NaCl
- 2g KCl
- 14,4g Na2HPO4
- 2,4g KH2PO4

Protocol

1. Dissolve all the reagents in 800ml of dH2O (or MilliQ)
2. Adjust pH to 7,4
3. Adjust volume to 1L with additional dH2O (or MilliQ)
4. Sterilize by autoclaving
5. Store at room temperature

## 7.7. Molecular markers, primers, PCR conditions and culture origins

**Table 24** - Description of the primers used for DNA sequencing of the cultures.

| Molecular Region | Molecule size (bp) | Forward Primer | Primer Sequence (5´-3´ direction) | Reverse Primer | Primer Sequence (5´-3´ direction) | Reference |
|---|---|---|---|---|---|---|
| 18S | ≈ 750 | SS5 | GGTTGATCCTGCCAGTAGTCATATGCTTG | SSE21.6 | CTAGAAACCAACAAATAGAACTGAGGTC | Santos et al. (2003) |
| 18S | ≈1800 | SS5 | GGTTGATCCTGCCAGTAGTCATATGCTTG | SS3 | GATCCTTCCGCAGGTTCACCTACGGAAACC | Rowan & Powers (1991) |
| ITS | ≈ 750 | ZITSUP | CCGGTGAATTATTCGGACTGACGCAGTGCT | ZITSDN | CTGTTTACCTTTTCCTCCGC | Santos et al. (2001) |
| 28S | ≈ 900 | ls 1.5 | CGCTGAAATTAAGCATATAAGTAAG | ls1.3 | AACGATTTGCACGTCAGTATC | Wilcox (1998) |

**Table 25** – Primers used for DNA amplification and the respective protocol.

| Molecular region | Primer combination | PCR profile | Reference |
|---|---|---|---|
| 18S (≈ 750bp) | SS5 + SSE21.6 | 94°C 2:30 min<br>56°C 1 min<br>72°C 2 min<br>94°C 1 min<br>56°C 1 min<br>72°C 2:30 min<br>29X to step 4<br>72°C 8 min<br>Hold 4°C | Santos et al. (2003) |
| 18S (≈ 1800bp) | SS5 + SS3 | 94°C 2:30 min<br>56°C 1 min<br>72°C 2 min<br>94°C 1 min<br>56°C 1 min<br>72°C 2:30 min<br>29X to step 4<br>72°C 8 min<br>Hold 4°C | Rowan & Powers (1991) |
| ITS (≈ 750bp) | ZITSUP + ZITSDN | 94°C 2 min<br>94°C 30 sec<br>60°C 30 sec<br>72°C 45 sec<br>35X to step 2<br>72°C 5 min<br>Hold 4°C | Santos et al. (2001) |
| 28S (≈ 900bp) | ls1.5 + ls1.3 | 94°C 2:00 min<br>94°C 15 sec<br>52°C 20 sec<br>72°C 45 sec<br>29X to step 2<br>72°C 7 min<br>Hold 4°C | Wilcox (1998) |

**Table 26** – Consensus sequence of the first DNA amplifications of the cultures.

| Molecular marker | Consensus sequence | Clade |
|---|---|---|
| 28S | ACATATAAGTAAGCGGAGGAAAAGGAACTAAATAGGAT<br>TCCCTCAGTAATGGCGAATGAACAGGGATGAGCTCAG<br>GCTGGAAACCGATACATCTGTGTCGGGCTGTAGCCTGC<br>AGGCATAGCGCTATCGGCGGCTCGAGCGCAAGCCTCT<br>TGGAATAGAGCGTGTGCCCGGGTGAGAATCCTGCATTT<br>CGCTCGATGTCCGCTGTCCACAGTGCTTGTCCTGAGAG<br>TCACGCTCCTCGGAATTGGAGCGTAAATCAGGTGGTAA<br>GTTTCATCCAAAGCTAAATAGAAGCTCGAGACCAATAG<br>CAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTG<br>GAAAGAGAGTTAAAAGTGTTTGAAATTGCTGAGAGCGA<br>AGCGAACGGAGCCACATGTCGTGCTGAGATTGCTGTG<br>GGTCTTTGTGAGCCTTGAGCATGTAAGCGCAAGCTGAC<br>TGCTTATGTGTGAGCATTTACCCGCAGTGTTTCTCAGCA<br>TGCGAGTCATTAGCCACATTTGTTTGAGGATGGCCTTC<br>AGCTCACATGGTACACTTGTTAGTGTTCCTCTGTGCGG<br>TGTGGGACTCAGACAGGTGTGGCTTGTTGACCCAGTG<br>GTTTTTTTCGACCCGTCTTGAAACACGGACCAAGGAGT<br>CTAGCACATGTGCGAGCTCTCGGGTAGCAAGCCTGAA<br>GGCGGAATGAAGGTGACTGCTGGGAATTGGCACCAGC<br>AACCGACCGATCAATTGGGAGAAGTTTGAGTTTGAGCA<br>TGTGTGTTAGGACCCGAAAGATGGTGAACTATGCCTGC<br>GAAGGGCAAAGTCAGGGGAAACTCTGATGGAGGCTCG<br>TAGCGA | B |
| ITS | TTCGGACTGACGCAGTGCTCAGCTTCTGGACGTTGTGT<br>TGGAAAGTTTCATGAACCTTATCACTTAGAGGAAGGAG<br>AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG<br>GATCATTCGCACTGATCAACTCTCGACACTGTGACTTTT<br>GATGTGAGGGACGCTTGTGGTGAAATATCTGCTTGCGG<br>AACTCGTGGGTGACAGCGGCGCATCGGCGCCTGGTGC<br>ATCTGGCACCAGGCGCGGGTGTTGCCCTGTGGCCGGG<br>TTAGCTCTAGGGTGAGATAGTTAGCGCAAGCTTTCTGG<br>AAAGGATGATGGTCCAAAACTACAACTTTCAGTGATGG<br>ATATCTTGGCTCGGGCACCTTTGAAGGGCGCAGCGAA<br>GCGCGATAGTCTTTGTGAATTGCAGAACTCCGTGAACC<br>GATGGCCTCCTGAACGCGCATTGCGCTCTCGGGATTTC<br>CTGAGAGCAGGTCTGCTTCAGTGCTTAGCATTATCTAC<br>CTGTGCTTGCAAGCAGCATGTATGTCTGCATTGCTGCT<br>TCGCTTTCCAACAAGTCATCGATCGCTTTTGTGTTCGTA<br>AATGGCTTGTTTGCTGCCTGGCCCATGCGCCAAGCTTG<br>AGCGTACTGTTGTTCCAAGCTTTGCTTGCATCGTGCAG<br>CTCAAGCGCGCAGCTGTCGGGATGCTGATGCATGCCC<br>TTAGCATGAAGTCAGACAAGAGAACCCGCTGAATTTAA<br>GCATATAAGTAAGCGGAGGAAAAGGGTAAACAG | B1 |

**Table 27 -** Cultures from the Warner lab with more than 1 strain per ITS2-type

| Culture Number | Original Host | Host Geographic Origin | Type |
|---|---|---|---|
| 61 | *Cassiopeia xamachana* | Florida | A1 |
| 362 | *Cassiopeia andromeda* | Gulf of Aquaba | A1 |
| 370 | *Stylophora pistillata* | Gulf of Aquaba | A1 |
| 23 | *Bartholomea annulata* | Barbados | A2 |
| 24 | *Bartholomea annulata* | Barbados | A2 |
| 25 | *Bartholomea annulata* | Barbados | A2? |
| 89 | *Gorgonia ventallina* | Bermuda | A2 |
| 97 | *Gorgonia ventallina* | Puerto Rico | A2 |
| 99 | *Gorgonia ventallina* | Puerto Rico | A2? |
| 104 | *Heliopora sp.* | Enewetak | A2 |
| 105 | *Heliopora sp.* | Enewetak | A2? |
| 107 | *Heliopora sp.* | Enewetak | A2? |
| 108 | *Heliopora sp.* | Enewetak | A2? |
| 130 | *Meandrina meandrites* | Jamaica | A2 |
| 154 | *Discoma santi-thomae* | Jamaica | A2? |
| 185 | *Zoanthus sociatus* | Jamaica | A2 |
| Culture X | - | - | A2 |
| M. faveolata | *Montipora faveolata* | Florida | A2 |
| M. miribilis | *Montipora miribilis* | - | A2 |
| PHMS TD1E A3A | - | - | A2 |
| 379 | *Plexaura homamala* | Bahamas | A4 |
| FLAP1 | *Aiptasia sp.* | Florida Keys | A4 |
| 2 | *Aiptasia pallida* | Florida | B1 |
| 12 | *Aiptasia tagetes* | Puerto Rico | B1 |
| 13 | *Aiptasia tagetes* | Bermuda | B1 |
| 74 | *Cassiopeia xamachana* | Jamaica | B1 |
| 146 | *Oculina diffusa* | Bermuda | B1 |
| 147 | *Pseudoterogorgia bipinnata* | Jamaica | B1 |
| M. capitata | *Montipora capitata* | Hawaii | B1 |
| 203 | *Hippopus hipopus* | Palau | C2 |
| HH1B C2 | - | - | C2 |

The list contains a total of 31 cultures from three clades and five ITS2-types. Type designations with a "?" were not entirely clear.